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CHARACTERIZATION OF THE NATURE OF OXIDANT ACTION USING MORPHOLOGICAL CHANGES OBSERVED FOLLOWING EXPOSURE OF *GIARDIA LAMBLIA* TO OZONE AND CHLORINE

BY

YANNING LI ©

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ABSTRACT

Morphology of *Giardia lamblia* cysts following single or sequential treatment with ozone and free chlorine was studied. Experiments were conducted in oxidant demand-free phosphate buffer using *in vitro* derived *G. lamblia* cysts. Nucleic acid staining using LIVE/DEAD BacLight™ was used for the evaluation of cyst viability. Examination of the ultrastructure of cysts was carried out using transmission electron microscopy. Cyst wall damage, plasmalemma breakage, lysis of peripheral vacuoles, nuclear degradation and other internal organelle damages illustrated the cyst death process elicited by oxidants. Inactivation by ozone and by free chlorine differed in the nature their damages to the endomembrane system, the nuclear structure and the cytoplasm. Cysts exposed to multiple chemical oxidants showed damages characteristic of changes elicited by both chemicals. Greater cytoplasmic damages were observed in cysts treated with free chlorine followed by ozone than cysts treated with ozone followed by free chlorine.

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DEDICATION

To my mom, dad and brother Da

for their love and support

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1. INTRODUCTION

As a result of untreated surface water, contaminated water distribution systems, and treatment deficiencies, many cases of waterborne outbreaks of protozoan infections have occurred in North America. *Giardia lamblia* is recognized as the most common cause of waterborne disease in North America (Craun and Calderon 1999; Craun *et al.* 2002; Kappus *et al.* 1994; Lengerich *et al.* 1994). Under the Surface Water Treatment Rule (SWTR), the USEPA requires 3.0 log-units removal of *Giardia* spp. cysts from the surface water supplies. Investigation of chemical inactivation of *Giardia* spp. concluded that ozone is the most effective chemical for the inactivation the *Giardia* spp. (Jarroll 1988) and that chlorine has limited effect on cyst viability. Free chlorine, the chemical disinfectant of choice for many years, has reported CT values for inactivation of *G. lamblia* that are approximately 100 times higher than those reported for ozone. The CT factor is defined as the product of the residual oxidant concentration, C, in mg/L, and the contact time, T, in minutes that residual oxidant is in contact with the water. Studies of sequential treatment showed that some chemical combinations were very effective for inactivation of protozoan cysts such as *Giardia* spp. and *Cryptosporidium parvum* (Bradbury 1998; Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c).

Despite the numerous investigations carried out on the disinfection of protozoan cysts, very little is known regarding the mechanism of inactivation of various oxidants. Such information would contribute significantly to the optimization of disinfection designs and treatment protocols. This study was undertaken to examine the ultrastructural changes in *G. lamblia* cysts following single or combined chemical inactivations. For single treatment processes, ozone and free chlorine were investigated. In the sequential treatment processes, ozone followed by free chlorine, and free chlorine followed by ozone were examined. The goal of this study was to characterize the nature of oxidant action using the morphological changes observed

following exposure of protozoa to ozone and chlorine. To achieve this goal, the following research objectives were established:

1. Examine the effect of low, moderate, and high treatment levels of ozone on the ultrastructure of *G. lamblia* cysts.
2. Examine the effect of low, moderate, and high treatment levels of free chlorine on the ultrastructure of *G. lamblia* cysts.
3. Determine the effect of ozone followed by free chlorine treatment of *G. lamblia* cysts.
4. Determine the effect of free chlorine followed by ozone treatment of *G. lamblia* cysts.

The *G. lamblia* cysts used in all experiments were obtained using *in vitro* encystation of *G. lamblia* WB trophozoites. Nucleic acid staining (SYTO-9) was used to measure cyst viability before and after treatment. De-ionized, phosphate buffered water was used in all microbial reduction studies.

2. LITERATURE REVIEW

2.1 Biology of *Giardia* spp.

2.1.1 Introduction

Giardia spp. is a flagellated unicellular eukaryotic microorganism that commonly causes diarrheal disease in humans and other animals throughout the world. It is recognized as the most common cause of waterborne disease in North America (Craun and Calderon 1999; Craun *et al.* 2002; Kappus *et al.* 1994; Lengerich *et al.* 1994). Giardiasis is an acute diarrheal illness. The infections can cause asymptomatic or chronic disease which is associated with recurrent diarrhea, malabsorption and weight loss (Wolfe 1990). The very young, the elderly and the immuno-compromised are particularly at risk for complications (Healy 1979; Lin 1985). Variability in clinical response to *Giardia* spp. infections may be contributed to differences in the host-parasite relationship (Janoff and Smith 1990).

The taxonomic position of *Giardia* spp. has been reviewed in detail (Levine 1979). The parasite is placed in the phylum *Sarcomastigophora*, class *Zoomastigophorea*, and the order *Diplomonadida*. Using the criteria of host specificity, morphology, and chemotaxonomy, *Giardia* spp. have been further classified into *G. agilis*, *G. muris*, and *G. lamblia* (Filice 1952; Meyer 1990). *G. lamblia* is also called *G. intestinalis* and *G. duodenalis*. *G. agilis* infects amphibians; *G. muris* infects rodents, birds, and reptiles; and *G. lamblia* infects some mammals including humans, as well as birds and reptiles.

The *Giardia* spp. life cycle (Figure 2-1) consists of an infective cyst and a vegetative trophozoite (Feely *et al.* 1990). The cyst is responsible for the infection of hosts and is transmitted through feces, fecally contaminated water or, less commonly, in food (Adam 1991). It is relatively resistant to harsh environmental conditions.

Excystation is triggered by exposure of cysts to gastric acid during their passage through the stomach and occurs after entry into the small intestine, where two trophozoites emerge from each cyst. The trophozoites penetrate the mucus layer to attach to the epithelium of the host villi by the ventral adhesive disc. They undergo mitotic division and may persist in the intestinal lumen for weeks to months. A certain number of trophozoites encyst. Release of cysts in the feces completes the life cycle.

2.1.2 Cell Biology

2.1.2.1 Trophozoite Structure

Morphology of *Giardia* spp. has been well documented (Feely 1979; Feely and Erlandsen 1985; Feely *et al.* 1980; Majewska *et al.* 1993). The *G. lamblia* trophozoites are pear-shaped and are approximately 12 to 15 μm wide. A median body, four pairs of flagella (anterior, posterior, caudal, and ventral), and a ventral disk make up the cytoskeleton. There are two nuclei without nucleoli located anteriorly. Organelles found in the cytoplasm include peripheral vacuoles, ribosomal and glycogen granules. The presence of Golgi complexes have been confirmed in encysting trophozoites but not in vegetative trophozoites. Golgi complexes have been suggested to exist based on stacked membrane structures in the cytoplasm.

The two nuclei in *G. lamblia* trophozoites appear nearly identical. Their replication is simultaneous as demonstrated by uridine incorporation into nuclear RNA (Wiesehahn *et al.* 1984). Both have equal numbers of genes and amounts of DNA as determined by *in situ* hybridization using the rDNA probe and the intensity of nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide, respectively (Kabnick and Peattie 1990). It is generally assumed that the two nuclei have the same complement of genes and chromosomes (Adam 2001). Unlike higher

eukaryotes, nuclei of *G. lamblia* do not have nucleoli. Transcription and processing of RNA, which normally occur in nucleoli of higher organisms, have been suggested to be distributed throughout the nucleus (Narcisi *et al.* 1998).

Trophozoites colonize the upper small intestine of their host by attaching to the intestinal wall with their concave ventral surfaces (ventral adhesive disk). The adhesive disc is very important for the survival of the organism in the intestine of the host where nutrients are obtained. The structure of the ventral disc has been described in detail (Feely *et al.* 1982; Friend 1966; Holberton 1973; Holberton 1981). The concave ventral adhesive disk covers the entire ventral surface. Components of the ventral disk include the bare area, lateral crest, and a more flexible ventrolateral flange. Contractile proteins responsible for the adherence include actinin, α-actinin, myosin, and tropomyosin (Feely *et al.* 1982). Active metabolism is required for attachment (Gillin and Reiner 1982). Inhibitory metabolic conditions such as temperatures below 37 °C, increased oxygen levels, and reduced cysteine concentrations will prevent attachment. Ultrastructurally, the disc is composed of repeating layers of uniformly spaced microtubules which form the base of microribbons that extend perpendicular from the membrane thus giving the striated appearance of the disc (Holberton 1973; Holberton 1981).

Studies have suggested that proteins of the disk including giardins, tubulins, and vinculins are involved in the attachment process (Narcisi *et al.* 1994; Peattie *et al.* 1989; Soltys and Gupta 1994). It has been proposed that the mode of action of anti-*Giardia* drugs such as metronidazole involve interaction with these proteins (Katelaris *et al.* 1994; Morgan *et al.* 1993). Proteins called giardins line the edges of the microribbons but are not found in the microtubules (Peattie *et al.* 1989). Proteins called tubulins are not found in the microribbons but are found in the microtubules (Soltys and Gupta 1994). The microtubules of the ventral disk, as well as those of the flagella, are presumably composed of β-tubulin. Vinculin is a protein that binds actinin and mediates the attachment of actin filaments to membrane sites (Narcisi *et*

al. 1994). Its location in attachment regions of the ventral disk has led to the suggestion that it may be involved in the attachment process. Studies of anti-*Giardia* drug (such as metronidazole) action have shown to target these proteins (Chavez *et al.* 1992).

The trophozoite has four pairs of flagella protruding from the anterior, posterior, caudal, and ventral regions (Friend 1966). The flagella emerge from two sets of basal bodies that are near the midline and anteroventral to the nucleus. The flagellar axonemes contain nine pairs of microtubules encircling two microtubules. The flagella appear to be important for motility but not for attachment (Erlandsen and Chase 1974; Feely and Erlandsen 1981; Feely and Erlandsen 1982) contrary to Friend's (1966) hypothesis. In addition, their early emergence through the cyst wall during the process of excystation suggests their importance in excystation (Buchel *et al.* 1987).

G. lamblia trophozoites typically have two median bodies that are shaped like claw hammers. As a component of the cytoskeleton, the median body is positioned in the midline and dorsal to the caudal flagella. This structure, unique to *Giardia* spp., helps to define the morphologic characteristics of the different *Giardia* spp. (Filice 1952). The median body has been speculated to serve as the assembly site for microtubules to be incorporated into the ventral disk (Meng *et al.* 1996a).

In the cytoplasm of *G. lamblia*, elements of the rough endoplasmic reticulum, numerous cytoplasmic cisternae and tubular membrane elements, individual and clustered vesicles, numerous coated small vesicles, and microtubules, both free and in bundles have been imaged and described (Gillin *et al.* 1996). Further evidence confirming the presence of the endoplasmic reticulum includes the cloning and characterization of the signal recognition particle receptor (SR) which is localized in the cytoplasmic portion of the endoplasmic reticulum (Soltys *et al.* 1996) and three protein disulfide isomerase genes whose products are localized in the endoplasmic

reticulum (Knodler *et al.* 1999). Furthermore, the identification of an extensive membrane system labeled with antibody to BiP (chaperonins in endoplasmic reticulum involved in folding and transportation of proteins destined for secretion) clearly demonstrate the existence of the endoplasmic reticulum in *G. lamblia* (Soltys *et al.* 1996).

Standard microscopy has demonstrated Golgi complexes in encysting but not in vegetative trophozoites (Gillin *et al.* 1996; Reiner *et al.* 1990). More recently, transmission and freeze fracture electron micrograph of nitrobenzoxadiazole (NBD) ceramide-labeled log phase trophozoites demonstrated heavy staining in the perinuclear region in a pattern similar to that of the Golgi complex from other organisms (Lanfredi-Rangel *et al.* 1999).

Trophozoites have a layer of elliptical to tubular shaped vacuoles encompassing the periphery of the cell. These vacuoles vary in size from 100 to 400 nm and differ in their content as determined by the variation in their electron opacity (Friend 1966). Cheissin (1964) considered them to be mitochondria. Having only a single unit membrane surrounding the internal matrix without infolding of that membrane, Friend (1966) suggested that they were analogous to the mucocysts of other protozoa and served in the secretion of the cyst wall. Subsequent research suggested that these peripheral vacuoles serve the functions of endosomes and lysosomes in higher eukaryotes (Lanfredi-Rangel *et al.* 1998). Bockman (1968) demonstrated the uptake of exogenous ferritin by the vacuoles of *G. muris*, suggesting a role in the ingestion of foreign material. Endosomal properties of peripheral vacuoles of *G. lamblia* are indicated by their acidity, as shown by their uptake of acridine orange (Feely *et al.* 1991; Kattenbach *et al.* 1991). Lysosomal characteristics are indicated by a variety of hydrolase activities, such as acid phosphatase, proteinase, and RNase (Feely and Dyer 1987; Lindmark 1988; McKerrow *et al.* 1993). More recent research has suggested a continuity of these vacuoles with the endoplasmic reticulum (Lanfredi-Rangel *et al.* 1998; Soltys *et al.* 1996). Thus, the vacuoles appear to function as early

and late endosomes and lysosomes and may be functionally associated with the endoplasmic reticulum (Lanfredi-Rangel *et al.* 1998).

3.1.1.1 Cyst Structure

The organelles and structures found in the trophozoite can also be found in the encysted organism, with some alterations (Feely 1988; Luchtel *et al.* 1980; Sheffield and Bjorvatn 1977). Two or four nuclei (depending on the maturity of the cyst) are found at one pole of the cyst. Axonemes of the flagella are seen as linear structure by light microscopy and in section by transmission electron microscopy showing the familiar "9+2" arrangement of microtubules. The cytoplasm also contains profiles of the median body, segments of rough endoplasmic reticulum and basal bodies. The cytoskeleton of the ventral disc is found in fragments which may appear in stacks or crescent. The periphery is marked by an array or layer of vacuoles. These are approximately 400 nm in diameter and resemble the peripheral vacuoles of the trophozoite.

The *Giardia* spp. cyst wall enables the parasite to survive wide ranges of pH, temperature and osmotic pressure. This shell-like structure is 0.3 to 0.5 μm thick (Sheffield and Bjorvatn 1977) and consists of a complex of carbohydrate and peptides. The cyst wall consists of two components: a filamentous layer and a membranous layer. The membranous layer is composed of the outer and inner cyst wall membranes which are separated by a thin layer of cytoplasm (Erlandsen *et al.* 1989; Erlandsen *et al.* 1990).

Four major proteins have been identified in the outer cyst wall, 29, 75, 88 and 102 kDa in size (Erlandsen *et al.* 1990). It has been demonstrated, using gas chromatography and mass spectrometry, that purified and isolated filamentous cyst walls of *Giardia* spp. cysts are composed of approximately 43 percent carbohydrate by dry weight (Manning *et al.* 1992; Jarroll *et al.* 1989a). The sugar component of

the outer portion is predominantly galactosamine in the form of N-acetylgalactosamine (Jarroll *et al.* 1989b). This polysaccharide accounts for many cyst wall properties, such as its physical strength and resistance to chlorine and other chemical agents (Aley and Gillin 1995). Perez *et al.* (1995) showed that N-acetyl glucosamine, a simpler sugar, was resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacted relatively fast with ozone, but glucose was relatively resistant to degradation. This observation may explain, at least in part, the higher resistance of protozoa such as *Giardia* spp. to ozone.

In addition to its function as a protective layer, the cyst wall must also play a role in signal transduction to be able to recognize and respond to external stimuli such as those triggering excystation (Gillin *et al.* 1996; Meng *et al.* 1994).

2.1.3 Encystation

2.1.3.1 Events and Morphology of Encystation

Recent success in the encystation of *G. lamblia in vitro* has contributed significantly to the study of the encystation process (Campbell and Faubert 1994; Faubert *et al.* 1991; Gillin *et al.* 1988; Gillin *et al.* 1991; Lujan *et al.* 1997; McCaffery and Gillin 1994; Reiner *et al.* 2001; Reiner *et al.* 1990; Schupp *et al.* 1988a). Formation of the *G. lamblia* cyst involves a series of events:

1) The expression of encystation-specific genes, such as those necessary for the synthesis and processing of cyst wall components;

2) The intracellular synthesis and transport of cyst wall components in form of secretory granules;

3) The release of secretory granule content and assembly of the extracellular cyst wall.

Biogenesis of organelles in trophozoites is an early event. Appearance of the Golgi-like stack of membranes as detected readily by electron microscope is followed by

the formation of encystation specific vesicles (Reiner *et al.* 1990). Monoclonal antibody labeling of protein initially occurred in encystation specific vesicles; its subsequent occurrence in the cyst wall demonstrates transport of cyst wall building blocks to the cyst wall via encystation specific vesicles (McCaffery *et al.* 1994; Ward *et al.* 1990). Two cyst wall proteins, a 26 kDa CWP1 and a 39 kDa CWP2 have been cloned and characterized. Expression of BiP, the molecular chaperone found in the endoplasmic reticulum, increases during encystation (Lujan *et al.* 1996).

In the late phase of encystation, cyst wall filaments appear on certain sites of the trophozoite plasmalemma. A set of larger proteins are expressed in addition to the lower-molecular-mass proteins (Reiner *et al.* 1989). Completion of cyst wall assembly is marked by a cessation of cyst motility and the disappearance of encystation specific vesicles. The encysted organisms contain two trophozoites with four nuclei that have not yet completed cytokinesis.

2.1.3.2 Promoting and Inhibiting Factors

Encystation of trophozoites takes place in the jejunum after exposure to biliary secretions. Specific conditions that promote encystation included a slightly alkaline pH, conjugated bile salts plus fatty acids, and deprivation of cholesterol.

The factors influencing the encystation of *G. lamblia in vitro* have been studied in detail (Gillin 1987; Gillin *et al.* 1987; Gillin *et al.* 1989; Gillin *et al.* 1988; Kane *et al.* 1991; Schupp *et al.* 1988b). Encystation *in vitro* can be triggered by exposing trophozoites to factors characteristic of the small intestine where encystation normally takes place *in vivo*. Incubation of trophozoites in slightly alkaline growth medium containing bile salts allows the production of cysts in concentrations as high as 10^5 per mL of encystation medium, with a viability of about 50 percent (Gillin *et al.* 1989; Kane *et al.* 1991). The optimum pH has been reported to be 7.8 (Gillin *et al.* 1988; Kane *et al.* 1991; Schupp *et al.* 1988b). The principle bile salts used were

porcine and bovine bile salts (Gillin 1987; Gillin *et al.* 1987; Gillin *et al.* 1989; Gillin *et al.* 1988; Kane *et al.* 1991; Schupp *et al.* 1988b). High pressure liquid chromatography analysis showed that porcine bile mainly consist of hyocholate, while bovine bile had less chenodeoxycholate and more deoxycholate than human bile (Gillin *et al.* 1986). Small amounts of porcine bile have been used to induce encystment of several strains of *G. lamblia* (Campbell and Faubert 1994; Gillin *et al.* 1989). However, the same method yielded minimal quantities of cysts by other groups (Kane *et al.* 1991; Schupp *et al.* 1988b). Instead, bacterial grade bovine bile triggers the encystation of large numbers of viable cysts (Kane *et al.* 1991; Schupp *et al.* 1988b).

Although a high pH and increased bile concentration can induce the production of cysts *in vitro*, the actual production of viable cysts is influenced by other factors. Lactic acid, a major product of bacterial metabolism in the human colon was found to increase viability when used with porcine bile, but not with bovine bile (Gillin *et al.* 1989). Growth of the pre-encystation culture without bile also increased viability. Earlier studies have shown that the storage of isolated *G. lamblia* cysts at 37 °C greatly reduced their viability (Bingham *et al.* 1979a). This elevated temperature, together with proteases from trophozoite lysis and high bile concentrations are all harmful effects of culture conditions. By exposing trophozoites to encystation stimulus for a short period of time and returning them to normal growth medium, Kane (1991), was able to greatly increase the viability of the *in vitro*-derived cysts.

2.1.4 Excystation

2.1.4.1 Events and Morphology of Excystation

Morphological changes as seen by scanning electron microscopic and transmission electron microscopic examinations during the excystation process have been described by Buchel and colleagues (Buchel *et al.* 1991a; Buchel *et al.* 1987) for *G.*

lamblia and by Coggins and colleagues for *G. muris* (Coggins and Schaefer 1984; Coggins and Schaefer 1986). The excystation process takes less than 10 minutes to complete. Following exposure to conditions that promote excystation, the caudal flagella and distal ends of the other flagella protrude from one end of the cyst. In induced cysts, peripheral vacuoles appear to release their contents into the peritrophic space. The trophozoites retracted from the cell wall, which resulted in a progressive enlargement of the peritrophic space (Buchel *et al.* 1991b). Buchel *et al.* (1991b) also observed a detachment of the outer cytoplasmic membrane of the cyst wall, followed by its breaking and formation into numerous small vesicles lodged between the wall and the organism. The cyst wall loses its tight arrangement of the microfibrils. The preventral flange enlarges as the emerging trophozoite separates from the cyst wall. Flagellar movement starts slowly but rapidly increases within the first five to ten minutes of their emergence. The rapid flagellar movement seems to help pull and break the trophozoite out of the cyst wall. Scanning electron microscopy of this process has shown a tear in the cyst wall of *G. muris* cysts from the polar opening toward the opposite pole (Coggins and Schaefer 1984). This opening appeared to be subsequently enlarged, presumably by flagellar action. Externally, the emergence of flagella through the opening cyst wall has followed by the entire trophozoite. Newly emerged trophozoites appear disorganized, oval in shape, and associated with mucoid-like material. During encystation, karyokinesis takes place which eventually results in a trophozoite with four nuclei and eight flagella. At the completion of excystation, trophozoites quickly begin to flatten, elongate, and undergo cytokinesis, resulting in 2 trophozoites each with 2 nuclei and completely organized adhesive disks (Buchel *et al.* 1987).

2.1.4.2 Promoting and Inhibiting Factors

In the mammalian host, excystation is triggered upon exposure to the contents of the upper small intestine after passage through the acidic environment of the stomach. Induction of excystation of *Giardia* spp. cysts *in vitro* was done initially by an acidic

pH (Bingham and Meyer 1979). A pH of 1.3 to 2.7 provided the optimum excystation condition. Subsequent studies demonstrated the induction of excystation of *G. muris* cysts in alkaline conditions (pH 7.5), refuting the necessity of acidic pH in excystation (Feely *et al.* 1991). The inhibition of excystation by 4-4'-diisothiocyanatostilbene-2-2'-disulfonic acid (DIDS) indicated the importance of vacuolar acidification in excystation. Optimum pH was found to be 4.0.

In addition to pH, enzymatic activities play an important role in the excystation process. Excystation was facilitated by pancreatic proteases and inhibited by a trypsin inhibitor, suggesting the importance of proteases in excystation (Schaefer *et al.* 1984). Intracellularly, a *G. lamblia* cysteine protease (CP2) of the cathepsin B family is required for excystation (Ward *et al.* 1997). Cysteine protease activity was found in the peripheral vacuoles. Inhibitors of cysteine proteases prevented excystation but did not affect trophozoite growth or replication. Recently, it was shown that protein kinase A plays an important role in cellular activation of excystation (Abel *et al.* 2001). In addition, certain calmodulin antagonists inhibited excystation, suggesting that calmodulin may be involved in excystation (Bernal *et al.* 1998). Excystation was inhibited by antibody to cyst wall and by wheat germ agglutinin (WGA), presumably by its reaction with one of more of the glycoproteins found in the cyst wall (Meng *et al.* 1996b; Meng *et al.* 1994).

2.1.5 Cyst Viability Assessment

2.1.5.1 Animal Infectivity

Viability of the *Giardia* spp. had been defined based on excystation rates, ability to uptake different fluorescent dyes, as well as efficiency of infecting host animals. Of all the methods only animal infectivity provides direct information about the ability of a particular parasite to infect the host. Animal models have been developed in the Mongolian gerbil (Belosevic *et al.* 1983) and the mouse (Roberts-Thomson *et al.*

1976). Mongolian gerbil was much more susceptible to infection with *G. lamblia* cysts than either rats or dogs. Cyst release pattern during of infection was intermittent - similar to that for human infection. In the Mongolian gerbil model, the probability of infection was directly related to the number of cysts administered. Logit response of gerbils was expressed as a function of the *G. lamblia* dose and the parameters for the logit model were developed using the least-squares method. The resulting model was:

$$\text{Logit} = -3.688 + 1.542 \log_{10} (\text{inoculum per animal})$$

where logit = Ln [P/(1-P)],

P = Proportion of the animal infected for the specified inoculum

Although being the most relevant measurement of viability, animal infectivity has many disadvantages. The test requires weeks to complete. Animals are expensive to buy, feed, and house. Also, only positive results can be conclusively used in animal infectivity studies. Just because an infection did not take place in the gerbil does not mean that the cysts used were not alive or infective to humans.

2.1.5.2 In vitro Excystation

In vitro excystation is a common method currently used to determine the viability of *G. lamblia* cysts. *In vitro* excystation techniques attempt to mimic the conditions of *in vivo* digestion. The cysts excyst in response to proteolytic digestion (usually in the presence of bile salts) resulting in the subsequent release of infectious trophozoites. Excystation methods vary depending on whether *G. lamblia* or *G. muris* are being excysted (Bingham *et al.* 1979b; Feely 1986; Rice and Schaefer 1981; Schaefer *et al.* 1984). For *G. lamblia*, the number of intact cysts (IC), partially excysted trophozoites (PET) and totally excysted trophozoites (TET) is indicative of the number of viable organisms present in the original suspension (Bingham *et al.* 1979b; Rice and Schaefer 1981). Viability can be determined by applying the following formula:

% excystation = (TET/2 + PET) + (TET/2 +PET + IC) x 100

The number of totally excysted trophozoites was divided by 2 because each excysted organism yielded a pair of trophozoites.

G. muris cysts can be routinely excysted with efficiencies in the 90th percentile (Feely 1986; Schaefer *et al*. 1984). *G. lamblia* excystation efficiencies, on the other hand, are erratic and usually much less than 90 percent (Schaefer 1990).

The USEPA recommended the use of *in vitro* excystation as the standard protocol for determining viability of *Giardia* spp. cysts. The use of excystation is preferred over that of animal infectivity because of the time and expense involved with animal models (Jarroll 1988). However, it has been reported in the literature that *in vitro* excystation often over-estimates viability and infectivity of cysts (deRegnier *et al.* 1989; Schupp and Erlandsen 1987).

2.1.5.3 Nucleic Acid Staining

Different vital dyes have been used for the assessment of *G. lamblia* cyst viability. Vital staining is based on the difference of cyst metabolic potential between live and dead parasites and is dependant on cyst wall permeability. The method offers advantages over excystation and infectivity in that individual cysts can be viewed independent of the host stimuli or the host. The techniques associated with both methods are relatively simple to use.

Early studies with vital dyes and parasites were conducted using eosin and were based on dye exclusion as a measurement of viability (Bingham *et al.* 1979b). The eosin dye exclusion technique always indicated a higher percent viability than indicated by excystation studies.

Fluorogenic staining has since replaced eosin as a reliable means of determining viability (Jones and Senft 1985; Schupp and Erlandsen 1987). The dye fluorescein diacetate, which permeates cell membranes, is enzymatically cleaved to release fluorescein in viable cells. Propidium iodide, which only enters damaged cells, is considered to be a marker for nonviable cells. Studies by several investigators have shown an excellent correlation between staining patterns with these two dyes and morphologic criteria used to determine *Giardia* spp. cyst viability (Hibler *et al.* 1987; Schupp *et al.* 1988b). There are unsatisfactory aspects to the fluorogenic dye technique. Fluorogenic dyes seem to correlate well with *G. muris* excystation but not with *G. lamblia* excystation.

Recently, SYTO-9 and SYTO-59 vital dye methods were developed for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts (Belosevic *et al.* 1997; Neumann *et al.* 2000; Taghi-Kilani *et al.* 1996), and were shown to correlate to animal infectivity. The use of fluorescent nuclei acid binding dyes as indicators of *Giardia* spp. and *C. parvum* viability has been examined. Surveys of single staining nucleic acid dyes (stain dead parasites only), and double staining vital dye kits from Molecular Probes® Inc (stain live and dead parasites) were conducted to assess the viability of untreated, heat killed, and chemically inactivated *G. muris* cysts. Nucleic acid staining results were compared to those of *in vitro* excystation and animal infectivity. A nucleic acid stain, designated as SYTO-9, was considered the best among the single staining dyes for its ability to stain dead cysts brightly and its relatively slow decay rate of visible light emission following DNA binding. SYTO-9 staining was correlated to animal infectivity. A Live/Dead BacLight(TM) was found to be the better of 2 double staining viability kits tested. Logarithmic survival ratios based on SYTO-9 and Live/Dead BacLight were compared to excystation and infectivity results for *G. muris* cysts exposed to ozone or free chlorine. The results indicate that SYTO-9 and Live/Dead BacLight staining are stable following treatment of cysts with oxidants.

An automated, flow cytometry-based nuclei acid staining viability assay has been developed for *C. parvum* oocysts, which is more reliable, highly reproducible, and significantly less time consuming. However, recent studies showed that SYTO-9 predictive models must be developed on a chemical disinfectant-specific basis, and limited to a given set of experimental conditions, because changes to parasite cell wall permeability to the SYTO-9 dye are dependent on the type of oxidant (Gyürék *et al.* 2001; Neumann *et al.* 2000). While SYTO-9 staining in conjunction with flow cytometry indicated correlation with animal infectivity in ozone or monochloramine treated *C. parvum* oocyst, it does not offer a robust *in vitro* assay for C. *parvum* oocysts treated with chlorine dioxide and free chlorine.

2.2 Chemical Inactivation of Microorganisms

2.2.1 Microorganism Reduction in Water Treatment

Water treatment employs a multi-barrier concept for efficient control of microorganisms. Watershed management reduces the number of microorganisms at the source; Physical processes such as coagulation, flocculation, sedimentation, dissolved air flotation and filtration remove a large number of pathogens; Disinfection forms the last barrier for protection of the public from waterborne pathogens.

Inactivation efficiency of an oxidant is indicated by the CT factor, a version of the Chick-Watson law (Chick 1908; Watson 1908). The CT factor is defined as the product of the residual oxidant concentration, C, in mg/L, and the contact time, T, in minutes that residual oxidant is in contact with the water. For a given inactivation level, a lower CT indicates higher disinfection efficacy.

Chlorine has been the most commonly used disinfectant in North America (Karazewski 1994). Stronger oxidants such as chlorine dioxide and ozone have been

used in some water treatment facilities (Graham *et al.* 1992; Lykins *et al.* 1994). More recently, the use of multiple chemicals has become a trend to further increase treatment efficiency. Design of chemical disinfection is influenced by disinfectant properties, economic factors, and public health issues such as treatment by-products.

2.2.2 Ozone

2.2.2.1 Ozone Chemistry

Ozone is widely used in disinfection today (Langlais *et al.* 1991; Rice 1991). It is generated by passing a high voltage electric discharge through dry air, and then absorbing the ozone in water. Ozone is characterized by a high oxidation potential and a high decomposition rate (Degrémont 1979).

Ozone reacts with various compounds directly or indirectly via radical species including hydroxyl free radical ($OH^{\cdot}$), ozonicle free radical anion (O^{3-}), superoxide free radical anion (O^{2-}), perhydroxyl free radical anion (HO_2^{-}) and hydrogen peroxide (H_2O_2) formed during ozone decomposition (Bryant *et al.* 1992; Glaze *et al.* 1987; Hoigné 1988; Hoigné and Bader 1978; Hoigné and Bader 1983).

Direct reactions tend to be highly selective and are often quite slow (minutes) (Hoigné and Bader 1979). Rate constants of direct reactions of ozone with various compounds are first order with respect to substrate and ozone concentrations (Hoigné and Bader 1983).

Indirect reactions of intermediate radical species formed during ozone decomposition are more complex. The highly reactive hydroxyl radical, with a life span of only a few microseconds in water, is believed to be the most important decomposition product (Glaze 1986; Glaze *et al.* 1987). Radicals formed in the decomposition

process can oxidize solutes or additionally accelerate the decomposition of ozone (Staehelin and Hoigné 1982).

Ozone decomposes either directly or in cycle (Tomiyasu *et al.* 1985). The sequence of reactions during cycle decomposition of ozone is initiation, propagation and termination (Glaze 1986; Hoigné and Bader 1983). Initiators are compounds that can react with ozone and produce a superoxide ion which will continue to react with ozone. In pure water the initiator is typically hydroxide ions. Other initiators include hydrogen peroxide, ferrous iron, natural organics, ultraviolet radiation and micro-organisms. Promoters, such as phosphate species, formic acid, primary alcohols and humic acids, are organic and inorganic molecules that can react with the hydroxyl radical to produce superoxide anion which reacts much more readily with ozone than with other aqueous solutes (Staehelin and Hoigné 1985). Inhibitors are defined as being compounds that consume hydroxyl radicals without regenerating superoxide anion and form relatively inert products (Staehelin and Hoigné 1982). Common inhibitors include bicarbonate and carbonate ions, tertiary alcohols and humic substances (Hoigné *et al.* 1985).

2.2.2.2 Disinfection Efficacy

Ozone is very effective against bacteria. Gram-positive bacteria, including *S. aureaus* and *Bacillus* spp., and the Mycobacteria are more resistant than are gram-negatives (Lee and Deininger 2000; Sobsey 1989). Ozone is generally more effective against vegetative bacterial cells than bacterial and fungal spores (Bablon 1991), but all are easily destroyed by relatively low levels of ozone.

Studies have reported a higher resistance to ozone by virus than vegetative bacteria (Bablon 1991; Sobsey 1989). Research has also shown bacteriophages being the least resistant to ozone, followed by polioviruses; human rotavirus is the most resistant to ozone (Hall and Sobsey 1993; Herbold *et al.* 1989).

Protozoan cysts are much more resistant to ozone. *Naegleria* spp. and *Acanthamoeba* spp. cysts are much more resistant to ozone than *Giardia* spp. cysts (Bablon 1991). *Cryptosporidium spp.* oocysts are the most resistant to ozone amongst protozoan cysts that have been studied (Langlais *et al.* 1990).

2.2.3 Chlorine

2.2.3.1 Chlorine Chemistry

Chlorine is considered as the most widely used disinfectant in the world (Craun *et al.* 2002). When chlorine dissolves in water the equilibrium below is rapidly established (White 1992):

$Cl_{2(g)} \Leftrightarrow Cl_{2(aq)}$

$Cl_{2(g)} + H_2O \Leftrightarrow HOCl + H^+ + Cl^-$

$HOCl \Leftrightarrow H^+ + OCl^-$

The species molecular chlorine (Cl_2), hypochlorous acid (HOCl), and hypochlorite ion (OCl^-) are all oxidizing agents collectively referred to as free chlorine. The speciation of chlorine depends on the pH of the water. Above pH 2, HOCl and OCl^- are the major chlorine species (Snoeyink and Jenkins 1980). Hypochlorous acid has pKa 7.5, therefore HOCl will predominate below pH 7.5 and OCl^- will predominate above pH 7.5.

2.2.3.2 Disinfection Efficacy

Chlorine has been shown to be very effective at inactivating bacteria (Butterfield *et al.* 1943). HOCl was shown to be more effective at inactivation of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Sheigella dysenteriae* than OCl^-.

Chlorine is also an effective oxidant against virus (Liu 1971). The study of 20 different enteric viruses at pH 5.8 and 2 °C found CT requirements of 1.4 to over 30 mg·min/L for inactivation of 4 log removal of virus of different resistance.

Chlorine is not very effective at inactivating protozoa. Research in disinfection of *Cryptosporidium* species reported that chlorine bleach solutions had little effect on oocyst viability (Campbell *et al.* 1982; Finch and Black 1994; Ransome *et al.* 1993; Sundermann *et al.* 1987). These studies determined that a 1 log removal of *Cryptosporidium parvum* at pH 6.0 and 22 °C required a CT level of 3000 to 4000 mg·min/L. It was suggested that these levels are not practical at typical water treatment doses of less than 5 mg/L (Korich *et al.* 1990).

2.2.4 Combined Disinfectants

Combined disinfection involves simultaneous or sequential application of two or more chemicals. The strong oxidant usually serves as the first disinfectant followed by the disinfectant with long lasting residual. Typical combinations in water treatment today include ozone followed by chlorine treatment, and free chlorine followed by monochloramine.

Initially, a second disinfectant was applied to maintain disinfectant residual in the water distribution system in order to control the bio-growth and provide a monitoring tool. However, researchers have found additional advantages to this practice. Many studies have observed synergistic effects on microorganisms when combined disinfectants were applied.

Simultaneous application of ozone and chlorine was found to be more effective than sequential application in inactivating *Salmonella typhimurium*, poliovirus type I, T2 coliphage and T3 coliphage in secondary wastewater and artificially polluted tap water (Kott *et al.* 1980). There have been other reports in the literature of synergism

between disinfectant species leading to more effective treatment. Sequential application of free chlorine followed by monochloramine was found to be an effective viral inactivating agent (Berman *et al.* 1988). Ammonium sulfate was added to free chlorine to form monochloramine. For free chlorine pretreatment involving contact times of less than 0.5 minutes, the levels of MS2 coliphage inactivation were greater than those attributable to the effect of chlorine pretreatment alone. Another study investigated *Escherichia coli* inactivation in a continuous flow system that permitted the stable coexistence of free chlorine and monochloramine (Kouame and Haas 1991). A potential mechanism of synergism was hypothesized to be sublethal injury caused by free chlorine which resulted in enhanced sensitivity to monochloramine. A study on the inactivation of total coliforms, fecal coliform, fecal streptococci, and coliphages using a combination of chlorine dioxide and chlorine indicated enhanced inactivation levels (Katz *et al.* 1994).

The effect of sequential application of disinfectants on the viability of *C. parvum* and *G. muris* has been studied. Significant synergy was measured when the encysted parasites were treated with ozone followed by chlorine, ozone followed by monochloramine, chlorine followed by monochloramine and chlorine dioxide followed by monochloramine (Finch *et al.* 2000a; Li *et al.* 2001b).

2.2.5 Factors in Microorganism Inactivation

2.2.5.1 Temperature

The effects of temperature on the inactivation of microorganisms are multi-faceted as it affects both the rate of reaction as well as the stability of chemicals. According to the van't Hoff-Arrhenius relationship, increasing temperature increases the rate of reaction between the chemical disinfectant and microorganisms. At the same time, the increase in temperature results in faster decomposition of the oxidants which is particularly true for ozone. Nonetheless, studies have shown that the effects of

increased reaction rate far exceed that of increased decomposition (Farooq *et al.* 1977b). Greater levels of inactivation were observed for ozonation of *Mycobacterium fortuitum* at higher temperatures with the same applied ozone dose, even though the residual ozone levels were lower at the higher temperatures (Farooq *et al.* 1977b). The change in resistance to ozone with temperature appears to vary depending on the organism. Resistance of *G. lamblia* cysts to ozone at 5 °C was nearly three times greater than that at 25 °C (Wickramanayake *et al.* 1984b). *N. gruberi* was about five times more resistant than *G. muris* at 25 °C, but only two times more resistant at 5 °C. In the case of *Cryptosporidium parvum*, ozonation at warmer temperature (22 °C) gave a higher degree of inactivation than was observed for experiments at 7 °C (Finch *et al.* 1993a).

In contrary to these findings, the effect of temperature on ozone inactivation of MS2 coliphage and HPC bacteria using a factorial experimental design demonstrated that lowering the temperature from 22 °C to 4 °C did not have a significant effect on the inactivation results (Helmer and Finch 1993). The inactivation rates for poliovirus 1 remained the same when the temperature was raised from 10 °C to 20 °C (Herbold *et al.* 1989).

2.2.5.2 pH

The effect of pH on the inactivation of microorganisms is also complex as it affects the characteristics of the disinfectant as well as properties of the microorganism.

Changes in pH affects speciation of chemicals and therefore results in changes disinfectant concentrations. Ozone decomposition increases as the pH increases (Sullivan and Roth 1980). Since OH^- is an initiator of ozone decomposition, alkaline pH results in less ozone being available (Forni *et al.* 1982). At pH less than 4, ozone decomposition is independent of pH (Hewes and Davison 1971) and is relatively slow at pH ranging from 4 to 6. However, decomposition becomes significant for pH

values ranging from 6 to 9.5 (Gurol and Singer 1982; Hoigné 1988). However since ozone disinfection reactions are usually rapid, disinfection may be achieved even under high pH conditions. Disinfection studies involving various microorganisms have indicated that differences in ozone inactivation kinetics at different pH conditions can be attributed to the rate of ozone decomposition (Langlais *et al.* 1991). Several studies that reported no difference in inactivation levels with pH changes all had ozone residual levels maintained constant, thus making ozone decomposition irrelevant. Farooq *et al.* (1977a) reported that ozone inactivation kinetics of *Mycobacterium fortuitum* did not significantly differ in the pH range of 6-10, at 24 °C, for constant ozone residual. Katzenelson (1979) observed that inactivation kinetics of poliovirus type 1 by ozone in ozone demand-free water at 4 °C did not differ significantly from pH 3 to 10. The inactivation of *Naegleria gruberi* cysts at pH 6, 7, and 8 was reported to not differ appreciably compared with that observed at pH 7 given the same constant ozone residual at 25 °C (Wickramanayake *et al.* 1984b). The inactivation of *Cryptosporidium parvum* was not found to be different from pH 6 to 8 given the same ozone residual (Gyürék *et al.* 1999).

The speciation of chlorine depends on the pH of the water. Significant concentration of free chlorine molecules will only be present in solution below about pH 1, and hence are almost absent at the pH of drinking water. Hypochlorous acid has pKa 7.5, therefore HOCl will predominate below pH 7.5 and OCl^- will predominate above pH 7.5. This is significant because HOCl is a more effective disinfectant than OCl^- (Fair *et al.* 1948). The difference in efficacy is probably because the neutral HOCl molecule can penetrate the cell membrane of microorganisms more easily than the ionic OCl^- (Chang 1944; White 1992). As a result, disinfection efficiency of chlorine increases with decreasing pH. Consequently water at pH greater than 7.5 requires a higher dose of chlorine or longer contact time to achieve a specified level of disinfection.

2.2.5.3 *Turbidity, Organic and Inorganic Compounds*

In general, dissolved organic compounds and reduced inorganic species such as iron and manganese lead to decreases in disinfectant efficacies by decreasing disinfectant residuals. The instantaneous oxidant demand observed within seconds following application of disinfectants can be attributed to reactions with sulfite, nitrite, olefinic aliphatic hydrocarbons, phenols, polyaromatic hydrocarbons, organic amines, and sulfides (Hoigné 1988; Hoigné and Bader 1994).

Bicarbonate and carbonate ions may be important to the inactivation of microbes because they act as inhibitors to ozone decomposition by reacting with intermediate free radicals. This means that more ozone will become available for more selective reactions and that less OH radical-induced oxidation will occur (Bablon 1991). Bicarbonate and carbonate ions can extend the life of ozone in water but at the same time it is most likely that they are going to have a negative effect on the oxidation rates of organic compounds (Doré *et al.* 1987). Removal of carbonate ions from groundwater (pH 7.7) reduced the half-life of ozone from 20 to 2 minutes even when the pH was kept constant by adding a buffer (Hoigné and Bader 1994).

Organic and inorganic compounds in water may affect disinfection efficiency by altering other properties of the microorganisms. These compounds have been shown to shield microorganism from disinfection processes (Herson *et al.* 1987). Association with organic and inorganic components often results to clumping and aggregation of microorganisms which lead to increased resistance to disinfectants (Chen *et al.* 1985; Clark *et al.* 1994).

2.2.5.4 *Disinfection By-Products (DBPs)*

Chemical disinfectants and their by-products are potential health hazards to the public, especially when applied at high doses. A maximum residual disinfectant level (MRDLs) for chlorine and chloramine has been set by the U.S. Environmental

Protection Agency (1998) at 4.0 mg/L. Chlorine reacts with organic compounds such as fulvic and humic acids to produce chlorinated organics such as trihalomethanes (THMs) and haloacetic acids (HAAs), both of which are recognized suspected cacinogens (Bull *et al.* 1995; White 1992). Maximum level of total THMs and HAAs have been set by the U.S. Environmental Protection Agency (1998) at 0.080 mg/L and 0.060 mg/L, respectively.

Ozone by-products include oxidation by-products and brominated by-products (Singer 1993). Brominated by-products result as ozone oxidizes bromide to hypobromous acid. The hypobromous acid then becomes involved with substitution reactions with natural organic matter to produce brominated by-products (Bourgine *et al.* 1993; Ko *et al.* 1996; Siddiqui *et al.* 1998). The maximum contaminant level (MCL) for bromate ion has been set at 0.010 mg/L by the U.S. Environmental Protection Agency 1998).

2.3 Chemical Inactivation of *Giardia* spp.

2.3.1 Single Chemicals

2.3.1.1 Ozone

Investigation of chemical inactivation of *Giardia* spp. concluded that ozone is the most effective chemical for the inactivation the parasite (Jarroll 1988).

In a study using excystation as measurement of viability and maintaining a constant ozone residual, Wickramanayake *et al.* (Wickramanayake *et al.* 1984a; Wickramanayake *et al.* 1985) observed CT value of 0.17 mg·min/L at 25 °C for 2 log of inactivation of *G. lamblia*. A much higher CT of 0.8 mg·min/L to effect the same level of inactivation was observed (Finch *et al.* 1993b). The study was done using animal infectivity as a measurement of viability. As well, a continuous ozone

monitoring system was used to determine the integrated ozone residual concentration. The revised Hom model was developed for ozone inactivation of *Giardia* spp. at 22 °C:

G. muris	$\log (N/No) = -2.65\ C^{0.35}\ T^{0.52}$
G. lamblia	$\log (N/No) = -3.16\ C^{0.72}\ T^{0.24}$

The study also found that the average ozone residual was more important than contact time for the inactivation of *G. lamblia*; whereas both parameters are equally important in *G. muris*. Inactivation of *Giardia* spp. with ozone was temperature dependent. Resistance of *G. lamblia* cysts to ozone at 5 °C was nearly three times greater than that at 25 °C (Wickramanayake *et al.* 1984a).

2.3.1.2 Chlorine Species

Studies of inactivation of *Giardia* spp. have concluded that chlorine has limited effect on cyst viability. Free chlorine, the chemical disinfectant of choice for many years, has reported CT values for inactivation of *G. lamblia* that are approximately 100 times greater than those reported for ozone.

Free chlorine has also been demonstrated to be more effective at lower pHs at which hypochlorous acid predominates. Using *in vitro* excystation, Jarroll *et al.* (1981a) showed that inactivation of *G. lamblia* cysts exposed to free chlorine decreased as the pH rose from 6 to 8 for the same chlorine concentration, contact time and temperature. At 5 °C a chlorine concentration of 8 mg/L inactivated greater than 2.7 log of the cysts at pH 6 and 7 but required 30 min to achieve the same result at pH 8 (Jarroll *et al.* 1981a). In a similar study, *G. lamblia* cysts exposed to 2.5 mg/L of chlorine at 5 °C caused greater than 2 log inactivation after 30 min contact time at pH 6, whereas 60 min contact time was required at pH 7 and 8 (Rice *et al.* 1982). On the other hand, the reverse has also been reported where increased pH increased inactivation of

Giardia spp. cysts by chlorine (Leahy *et al.* 1987). Alteration to the surface of cyst walls at alkaline pH was considered a possible explanation to this anomaly (Rubin *et al.* 1989).

In regulation of the CT requirement for *Giardia* spp. inactivation by the U.S. Environmental Protection Agency, a factor of 2 increase in CT was used for every 10 °C decrease in water temperature within the range of 1 to 25 °C. Comparison of the CT products reported by (Wickramanayake *et al.* (1984a) indicates that the resistance of *G. lamblia* cysts to ozone inactivation at 5 °C is nearly 3 times more than that at 25 °C. Their resistance to chlorine appears to increase by approximately 1 order of magnitude as the temperature drops from 25 to 5 °C at pH 7 (Jarroll *et al.* 1981a). Thus, when compared to chlorine, ozone inactivation of *G. lamblia* cysts is less affected by temperature.

2.3.2 Multiple Chemicals

2.3.2.1 Ozone Pre-treatment

In-depth investigations on the effect of sequential treatment of various disinfectants on the inactivation of *Giardia* spp. and *Cryptosporidium parvum* have been carried out (Bradbury 1998; Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c). Sequential treatment by chemical compounds that provides more inactivation than that expected from the additive effects of single compounds demonstrates synergy.

Ozone has been shown to be one of the most effective chemicals for control of *Giardia* spp. However, due to ozone's rapid decomposition rate, chlorine or chloramine are often used as the secondary chemical treatment in large water systems. Previous studies showed that the sequential treatment using ozone followed by

chlorine species enhanced the inactivation of encysted parasites when compared to the same microbial reduction chemicals used alone (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c). Bradbury (1998) investigated the effect of multiple disinfectants on the inactivation of *G. muris*. At pH 6 and 8, ozone followed by chlorine showed a strong synergistic effect (Table 2-1). Ozone followed by monochloramine at pH 8 and pH 11 also showed significant synergy (Table 2-2).

Table 2-1. *G. muris* inactivation using ozone followed by free chlorine

Disinfectant	Level of Inactivation (log-units)		
	pH 6	pH 8	pH 11
Ozone	0.5	0.8	0.4
Free chlorine	0.8	0.6	0
Ozone followed by free chlorine	2.3	2.2	0.4
Inactivation attributed to synergism	1.0	0.8	0

Source: (Bradbury 1998)

pH 6: ozone 0.1 mg/L for 60 seconds; free chlorine 2.0 mg/L for 30 minutes.

pH 8: ozone 0.1 mg/L for 17 seconds; free chlorine 2.0 mg/L for 60 minutes.

pH 11: ozone 0.1 mg/L for 5 seconds; free chlorine 2.0 mg/L for 60 minutes.

Table 2-2. *G. muris* inactivation using ozone followed by chloramine

Disinfectant	Level of Inactivation (log-units)	
	pH 8	pH 11
Ozone	0.8	0.4
Monochloramine	0.5	0.7
Ozone followed by monochloramine	2.1	1.8
Inactivation attributed to synergism	0.8	0.7

Source: (Bradbury 1998)

pH 8: ozone 0.1 mg/L for 17 seconds; monochloramine 2.0 mg/L for 150 minutes.

pH 11: ozone 0.1 mg/L for 5 seconds; monochloramine 2.0 mg/L for 5 minutes.

2.3.2.2 *Chlorine Pre-treatment*

Studies on disinfection of *G. muris* using free chlorine followed by monochloramine suggested that a synergism exists involving the two chlorine species (Bradbury 1998) (Table 2-3). If chlorine inactivation were to be an additive phenomenon, the initial concentration and contact time of monochloramine required for a given level of inactivation would be the same regardless of the level of free chlorine pretreatment. Studies have shown that increasing levels of free chlorine pretreatment proportionally reduce the subsequent monochloramine concentration and contact time necessary for a given level of inactivation (Gyürék *et al.* 1996).

Table 2-3. *G. muris* inactivation by free chlorine followed by monochloramine

Disinfectant	Level of Inactivation (log-units)	
	pH 8	pH 11
Free chlorine	0.6	0
Monochloramine	0.5	0.7
Free chlorine followed by monochloramine	2.4	0.7
Inactivation attributed to synergism	1.3	0

Source : (Bradbury 1998)

pH 8: free chlorine 2.0 mg/L for 60 minutes; monochloramine 2.0 mg/L for 150 minutes.

pH 11: free chlorine 2.0 mg/L for 60 minutes, monochloramine 2.0 mg/L for 5 minutes.

Enhanced excystation following chlorine treatment has been reported and has been attributed to thinning, perforation, or complete removal of the outer layer of the cyst wall (Reduker and Speer 1985). The inner zone of the inner cyst wall was reportedly unaffected by sodium hypochlorite treatment. Other studies have shown hypochlorite treatment to sensitize *Clostridium botolinum* spores to germination by lysozyme. Scanning electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis reportedly indicated that spore coat proteins may be removed by hypochlorite treatment, increasing permeability to the cortex (Foegeding 1983).

Based on the review of the literature, chlorine pretreatment was postulated to affect the cyst wall sufficiently to alter the permeability but not to prevent infection of a suitable host. Subsequent treatment with monochloramine permits the oxidant to diffuse through the cyst wall and damage the trophozoites so that an infection cannot be initiated in a host. It is further postulated that the cyst wall permeability was likely to increase proportionally with respect to the level of chorine pretreatment, though there is probably a limit beyond which the benefit is minimal.

2.4 Mechanisms of Chemical Inactivation

2.4.1 Cell Death

Cell death has been classified into necrosis which is death resulting from pathological deviation in the environment, and apoptosis which occurs in more physiological circumstances (Wyllie 1974; Wyllie 1994). Necrosis shows cytoplasmic swelling and mitochondrial damage consistent with the hypothesis that it results from failure in osmotic regulation, caused by loss of cellular energy supplies. Apoptosis shows cytoplasmic condensation and prominent nuclear changes, the later closely associated with endonuclease activation. A recent review of cell death by Van Cruchten and Van den Broeck (2002) describes the different terminologies used in cell death studies. "Apoptosis is marked by cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane with eventually breaking up of the cell in apoptotic bodies. Cell death marked by cellular swelling should be called oncosis, whereas the term necrosis refers to the morphological alterations appearing after cell death. Apoptosis and oncosis are therefore pre-mortal processes, while necrosis is a post- mortal condition." In many cell types apoptosis fails to proceed in the absence of intact macromolecular synthesis. These observations are compatible with the hypothesis that apoptosis represents a cellular function activated from within. These two modes of death appear to account for most cell death in higher organisms, but in phylogenetically lower animals the connotations of necrosis may be different,

including homeostatic regulation, and morphological forms deviating somewhat from apoptosis may also exist (Wyllie 1974; Wyllie 1994). Since this study deals with disinfectant inactivation, the type of cell death is most likely characterized as necrotic cell death.

Morphology of necrosis has been reviewed by many (Arstila and Trump 1969; Dive *et al.* 1992; Ginn *et al.* 1969). Major findings are summarized in Figure 2-2. The earliest changes include a mild degree of cytoplasmic oedema, dilation of the endoplasmic reticulum, slight mitochondrial swelling, disaggregation of polysomes, and the appearance of multiple small aggregates of condensed chromatin around the nuclear periphery. These changes are probably all reversible. The point of no return to death is marked by high amplitude swelling of mitochondria (Ginn *et al.* 1969). Lethal changes that follow include extensive cytoplasmic swelling, dissolution of cytoplasmic organelles and rupture of plasma membranes. Lysosomes appear intact until cytoplasmic degradation is advanced. Few changes are made to the nucleus. The nucleus swells and the nucleoplasm becomes increasingly electron lucent. Small coarse chromatin masses scatter with the nucleus. It appears that the nucleus may rupture around the same time as the plasma membrane, or it may escape, with other organelles, when the plasma membrane bursts.

Since the first event in the morphological changes of necrosis is disruption of plasma membrane, it has been widely thought that the lethal action of a disinfectant is due to its capacity to damage the cell membrane, either by physically damaging it causing destruction or impairment of cellular structural organization or react with a protein, and more specifically, with the proteins of essential enzymes (Maris 1995; Sikkema *et al.* 1995). Many enzymes such as the dehydrogenases and permeases are either located inside the membrane or associated with it.

Alternatively, disinfectants could interfere with cellular activities (Jacangelo *et al.* 1991; Venkobachar *et al.* 1975). This can be done by inhibiting enzymes thus

inhibiting any metabolic pathways dependent on the enzymes inactivated. Enzymes control all metabolism including energy-yielding pathways and biosynthesis. However, even this mode of action is highly dependent on the membrane system. Uptake of molecules by the cell is dependent on the permeability of membranes, the transport proteins embedded within the membranes, and the metabolic processes within the membranes. Even if the primary site of lethal event is not the membrane itself, increasing cell sensitivity to toxic agents by increasing membrane permeability could cause much improved inactivation of cells.

2.4.2 Ozone Inactivation Mechanisms

2.4.2.1 Bacteria and Protozoa

2.4.2.1.1 Cell Membrane/Envelope

The first site of attack by ozone has been reported to be the cell membrane (Christensen and Giese 1954). Ozone may oxidize polyunsaturated fatty acids (Perchorowicz and Ting 1974), membrane-bound proteins (Goldstein and McDonagh 1975; McKersie *et al.* 1982; Mudd *et al.* 1969), glycoproteins and glycolipids (Scott and Lesher 1963; Murray *et al.* 1965) leading to leakage of cell contents and eventually causing lysis. Perez *et al.* (1995) showed that N-acetyl glucosamine, a compound present in the peptidoglycan of bacterial cell walls, in viral capsids, as well as in cyst walls of encysted protozoa, was resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacted relatively fast with ozone, but glucose was relatively resistant to degradation. This observation may explain, at least in part, the higher resistance of gram-positive bacteria compared to gram negative ones; the former contain greater amounts of peptidoglycan in their cell walls. This may also explain the high resistance of protozoa such as *Giardia* spp.

Several studies have documented physical damages from microscopic analysis of organelle and plasma membranes (Dave 1999; Khadre and Yousef 2001; Langlais

and Perrine 1986; Perrine *et al.* 1984; Swanson *et al.* 1977; Wickramanayake 1984). Dave (1999) found that treatment of *Salmonella enteritidis* with aqueous ozone disrupted the cell membranes as seen in TEM. Khadre and Yousef (2001) found that spores of *Bacillus subtilis* treated with aqueous ozone showed heavily disrupted outer spore coats. Microscopic observation of inactivation of trophozoites of *Naegleria* spp. and *Acanthamoeba* spp. showed that they were rapidly destroyed and the cell membrane was ruptured (Perrine *et al.* 1984). Langlais and Perrine (1986) showed that ozone affects the plugs in *Naegleria gruberi* cysts. Depending on the ozonation conditions, these plugs were completely removed or were partially destroyed. It has been speculated that ozone initially affects the *G. muris* cysts wall and makes it more permeable (Wickramanayake 1984). Subsequently, aqueous ozone penetrates into the cyst and damages the plasma membranes, additional penetration of ozone eventually affects the nucleus, ribosome, and other ultrastructural components. This hypothesis was supported by the observation of leached cytoplasm from *G. lamblia* cysts which led to speculation that ozone caused a progressive breakdown of the cyst wall, plasma membrane and cytoplasm (Wallis *et al.* 1990). Electronic microscopic analysis of organelle and plasma membranes has shown variation in membrane thickness after ozone treatment (Swanson *et al.* 1977).

Damage to the cell membrane was supported by reports of ozone induced increase in sensitivity to subsequent kill. Ozone treated bacteria cells *Acinetobacter anitratus* and *Pseudomonas aeruginosa* were reported to be more susceptible to complement-mediated killing (Doroszkiewicz *et al.* 1994). The authors concluded that ozone damage of cell membrane led to a more rapid penetration of the membrane attack complex of complement. Foegeding (1985) found that *Bacillus cereus* spores with coat proteins removed were rapidly inactivated by ozone, compared to intact spores. The researcher concluded that the spore coat is a primary protective barrier against ozone. Ozone was also found to alter proteins and fatty acid double bonds in the cell membrane of bacteria, adversely affecting permeability (Pryor *et al.* 1983).

2.4.2.1.2 Enzymatic and Metabolic Activities

Several authors referred to enzyme inactivation as an important mechanism by which ozone kills pathogen. Inactivation of enzymes by ozone involves the oxidation of sulfhydryl groups of enzymes (Chang 1971). Others have shown ozone-induced covalent intermolecular cross-linking of protein molecules as part of the membranes (Ignatenko and Cherenkevich 1985).

Sykes (1965) reported that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant. This may explain the difference between ozone and chlorine disinfection efficacies. In a study done by Whiteside and Hassan (1988) the antioxidant enzymes, catalase and superoxide dismutase, were inactivated after exposure to ozone. Takamoto *et al.* (1992) observed that ozone decreased enzyme activity in *Escherichia coli* at a greater degree in case of cytoplasmic α-galactosidase than in case of the periplasmic alkaline phosphatase. Ingram and Barnes (1949), in view of their finding general destruction of the dehydrogenating enzymes systems in the cell, proposed that ozone kills *Escherichia coli* by interfering with the respiratory system.

2.4.2.2 Viruses

2.4.2.2.1 Virus Capsid

Viruses are simple microorganisms composed of strands of RNA or DNA covered by a protein coat (capsid). Inactivation of virus can either occur through damage to the protein coat thereby preventing uptake by host cell, or though damage to the viral nuclei acid which makes the virus non-infectious.

Damage to the virus capsid as the lethal action by ozone has been demonstrated by many (deMik and deGroot 1977, Sproul and Kim 1980; Herbold *et al.* 1989; Kim *et al.* 1980; Kim *et al.* 1984; Riesser *et al.* 1977; Shinriki *et al.* 1988; Sproul *et al.* 1982; Yoshizaki *et al.* 1988). Studies showed that ozone damaged the protein capsid of

poliovirus type 2 (Riesser *et al.* 1977) and bacteriophage ϕX 417 (deMik and deGroot 1977). Subsequent result included an inhibition of uptake into the susceptible cells (Riesser *et al.* 1977) and breaks in the phage DNA (deMik and deGroot 1977). The same was also demonstrated in subsequent studies (Kim *et al.* 1980; Kim *et al.* 1984; Sproul *et al.* 1982). The authors found that aqueous ozone inactivated both f2 and T4 bacteriophages by attacking capsid protein, with liberation and inactivation of the nuclei acid. The RNA from f2 bacteriophage was partially inactivated prior to release from the capsid. They suggested that ozone breaks the protein capsid into subunits liberating RNA and disrupting virus adsorption to the host pili, and that the RNA may be secondarily inactivated. The DNA released from T4 bacteriophage was rapidly inactivated by ozone at about the same rate as that in the intact phage. Ozone randomly destroyed the head, collar, contractile sheath, and tail fibers of bacteriophage T4 and liberated the DNA from the head (Kim *et al.* 1984). Yoshizaki *et al.* (1988) found that aqueous ozone caused the coat proteins subunits of tobacco mosaic virus (TMV) to aggregate with each other and cross-link with the viral DNA. Despite their observation of a good correlation between loss of infectivity and decrease of recovery of viral RNA, Yoshizaki *et al.* (1988) and Shinriki *et al.* (1988) concluded that the major cause of TMV inactivation by ozone was the inability of the treated virus to uncoat. The observation (Herbold *et al.* 1989) that 0.38 mg/mL aqueous ozone was needed for complete inactivation of hepatitis A virus (HAV) and only 0.13 mg/mL for complete inactivation of poliovirus may support the hypothesis that damage to viral envelope is the main cause of inactivation of virus by ozone. Enveloped viruses such as HAV are expected to be much more resistant to ozone compared to non-enveloped viruses such as poliomyelitis.

2.4.2.2.2 RNA/DNA

Damage to the nucleic acid, which makes the virus permanently non-infectious is superior to damage that impairs only the protein coat of the virus. Some researchers have suggested this as the mode of ozone viricidal action. Christensen and Giese (1954) attributed the effect of ozone on nucleic acid to its action on the purines and

pyrimidines, each of which appeared to be affected. Roy *et al.* (1981) found that ozone altered two of the four polypeptide chains in the poliovirus protein coat. They, however, attributed the inactivation of the virus to the damage in its RNA by ozone.

2.4.3 Chlorine Inactivation Mechanisms

2.4.3.1 Bacteria and Protozoa

2.4.3.1.1 Cell Membrane/Envelope

Many studies on chlorine inactivation have implicated the disruption of the cell membrane as the lethal event causing inactivation. Morphological evidence showed damage to the cell membrane (Neuwirth *et al.* 1988; Zaske *et al.* 1980). Electron microscope examinations of injured *Escherichia coli* cells showed morphological changes in the cell envelope following chlorine treatment (Zaske *et al.* 1980). The morphology of *Giardia* spp. cysts following chlorine treatment was studied by Neuwirth *et al.* (1988) and Sauch and Berman (1991). TEM examinations of *G. muris* cysts treated with 4.3 mg/L chlorine for 20 minutes or greater showed morphological changes including "loss" of cell wall and enlargement of the lacunar space indicative of changes to the integrity of the cyst wall (Neuwirth *et al.* 1988). An immunofluorescence and morphology study of *G. lamblia* cysts exposed to chlorine between levels of 1.1 mg/L to 11.7 mg/L for up to 48 hours showed deterioration of internal features (Neuwirth *et al.* 1988). No conclusive evidence regarding damage to cyst wall could be obtained from the phase-contrast micrographs.

Permeability changes due to chlorine treatment have been documented by many (Chang 1944; Kulikovsky *et al.* 1975; Venkobachar *et al.* 1977; Williams *et al.* 1991). Chang (1944), in his study of *Entamoeba histolytica* cysts, found an increase in inactivation with decreasing pH. It was postulated that increased concentration of uncharged hypochlorous acid at lower pH's increase the disinfectant's permeability. Venkobachar *et al.* (1977) observed the leakage of proteins, RNA, and DNA

following treatment of *Escherichia coli* cells with very low doses of chlorine. DNA was found to be released at very high concentration of chlorine. The authors concluded that chlorine must have damaged the integrity of the cell membrane resulting in a leakage of macro and micro-molecules. Haas and Engelbrecht (1980) supported this in a study using *E. coli*. The authors showed that chlorine promoted cell leakage of potassium ions, total organic carbon, UV-absorbing material, in decreasing order of quantity. It was therefore concluded that lower molecular weight substances are preferentially released.

Similar results were seen in more resistant spore forming bacteria (Kulikovsky *et al.* 1975; Williams and Russell 1991). Chlorine treated *Bacillus cereus* spores released nucleic acids, which was believed to be a consequence of the destruction of the permeability barrier of the organism (Kulikovsky *et al.* 1975). Using the same organism, researchers observed efflux of potassium from the cells and a dramatic increase in hydrogen ion permeability following free chlorine treatment (Williams and Russell 1991).

2.4.3.1.2 Enzymatic and Metabolic Activities

Chlorine has been reported to interfere with metabolic processes associated with cell membrane. Inhibition of enzyme activity was documented as early as 1946 when Green and Stumpf (1946) reported that bacterial inactivation was due to the inhibition of certain enzymes. Since then, study shows that specifically the activity of sulfhydryl enzymes such as succinic oxidase, amino acid oxidase, transaminase etc. are inhibited; whereas non-sulfhydryl enzymes such as catalase and ATPase are not affected (Knox and Stumpf 1948; Venkobachar *et al.* 1975)

Inhibition of glucose oxidation by oxidation of sulfhydryl groups of essential enzymes was suggested to be the mechanism of inactivation by Green and Stumpf (1946) and Knox and Stumpf (1948). Studies involving hundreds of experiments on various bacteria found that chlorine was effective even at trace levels. This led to the

suggestion that chlorine must inhibit essential enzymes of an essential metabolic pathway such as glucose oxidation. It was shown that oxidation of sulfhydryl group by chlorine was instantaneous and irreversible. The authors suggested that insensitivity of spore cells to chlorine is not due to their impermeability to chlorine, but, rather, to the fact that the systems essential to viability are not the same as those of vegetative cells and are not as sensitive. Various enzymes were inhibited included tiosephosphate dehydrogenase (Green and Stumpf 1946), d-amino acid oxidase and transaminase (Knox and Stumpf 1948)

Other enzymes affected included dehydrogenase (Skidal'skaia 1969; Venkobachar *et al.* 1975). It was found that the total dehydrogeanase activity of intact *Escherichia coli* is affected by a chlorine dose that correlates with viability levels. The succinic dehydrogenase activity in the crude extract of *Escherichia coli* is decreased by chlorine within the bactericidal concentrations, and can be reversed with glutathione. More importantly, the authors observed a reduction in thiol group following chlorine treatment indicating vulnerability of the thiol groups to the oxidizing influence of chlorine. The ATPase, a non-sulfhydryl enzyme, also localized in the cytoplasmic membrane of cells, was not affected to any degree by chlorine in bactericidal concentrations (Venkobachar *et al.* 1975). Other non-sulfhydryl enzymes including catalase of *Escherichia coli* were not be inhibited by chlorine (Knox and Stumpf 1948; Venkobachar *et al.* 1975).

2.4.3.2 Viruses

2.4.3.2.1 Virus Capsid

Some researches showed that the modification of the viral protein coat, morphology of the viral particles and the permeability change of the outer protein coat, could be the mechanisms of viral inactivation by chlorine (Churn *et al.* 1983; Fauris *et al.* 1982; Tenno *et al.* 1979; Young and Sharp 1985). Churn *et al.* (1983) found covalently cross-linked capsid peptides in chlorine treated poliovirus. Treatment of the virus with chlorine did not cause any breakage in the nucleic acid, nor did it impair the

ability of the DNA to undergo *in vitro* replication. Electron microscopic examinations revealed that the DNA was extruded as a tail like structure which remained attached to the virus particle. The DNA was intact and still capable of *in vitro* replication. The adsorption of the chlorine-treated virions to host cells was inhibited. An alteration of the integrity of the virion capsid by rearranging, not degrading, the major polypeptides may be all that is required for inactivation. Tenno *et al.* (1979) demonstrated that inactivation of poliovirus by low concentrations of chlorine occurred before the nucleic acid lost its biological activity. Tenno *et al.* (1979) concluded that chlorine inactivated poliovirus by reacting with the protein component of the virus but that it does not result in any detectable change in the structure of the virus and does not affect the infectivity of the viral RNA.

2.4.3.2.2 RNA/DNA

Dennis *et al.* (1979) and Noss and Olivieri (1985) reported that the inactivation of f2 bacteriophage by chlorine was due to damage of the nucleic acid. Dennis *et al.* (1979) noted that chorine has an affinity for RNA at a pH of 5.6 – 9.9, which is greater than its affinity for protein. CMP and AMP consumed chlorine at low pH more actively than did GMP, with UMP being un-reactive. Preferential reaction between chlorine with RNA rather than with the capsid protein was also documented by Noss and Olivieri (1985) who studied chlorination of f2 bacteriophage.

In a study of chlorine effect on the polio virus, O'Brien and Newman (1979) reported degradation of RNA into fragments within the capsid and the subsequent release of these fragments. It was determined that virus inactivation occurred before release. RNA from intact virus exposed to chlorine was not altered (based on centrifugation). Empty capsids conformational state was not altered (pH 7.0).

2.4.4 Multiple Chemical Inactivation Mechanisms

Enhanced inactivation has been observed by recent studies using a combination of chemical disinfectants simultaneously or sequentially. There has been little investigation done on the mechanisms of synergistic inactivation. Two hypotheses have been made by researchers. Synergy can be the result of sub-lethal injury of microorganism induced by one chemical leading to an enhanced sensitivity to another chemical (Kouame and Haas 1991). Alternatively, synergy can be the result of increased ease of penetration of a second chemical through a cell membrane whose permeability has been increased by the first chemical (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c).

Kouame and Haas (1991) observed synergistic inactivation of *Escherichia coli* when exposed to free chlorine and monochloramine simultaneously. The authors suggested that free chlorine caused sub-lethal injury in *Escherichia coli.* As a result, the microorganisms were more susceptible to inactivation by monochloramine.

A series of in-depth studies were conducted on the effect of sequential treatment of *C. parvum* and *Giardia* spp. It has been observed that inactivation levels due to sequential treatment were higher than the additive effects of single disinfectant treatments (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c). Enhanced effectiveness of multiple disinfectants was hypothesized to be a conditioning of the oocyst wall of *Cryptosporidium* spp. by the first oxidant (i.e., chlorine, chlorine dioxide, or ozone) so that the secondary oxidant (i.e., chlorine, chlorine dioxide, and monochloramine) can penetrate the oocyst more easily (Liyanage *et al.* 1997c).

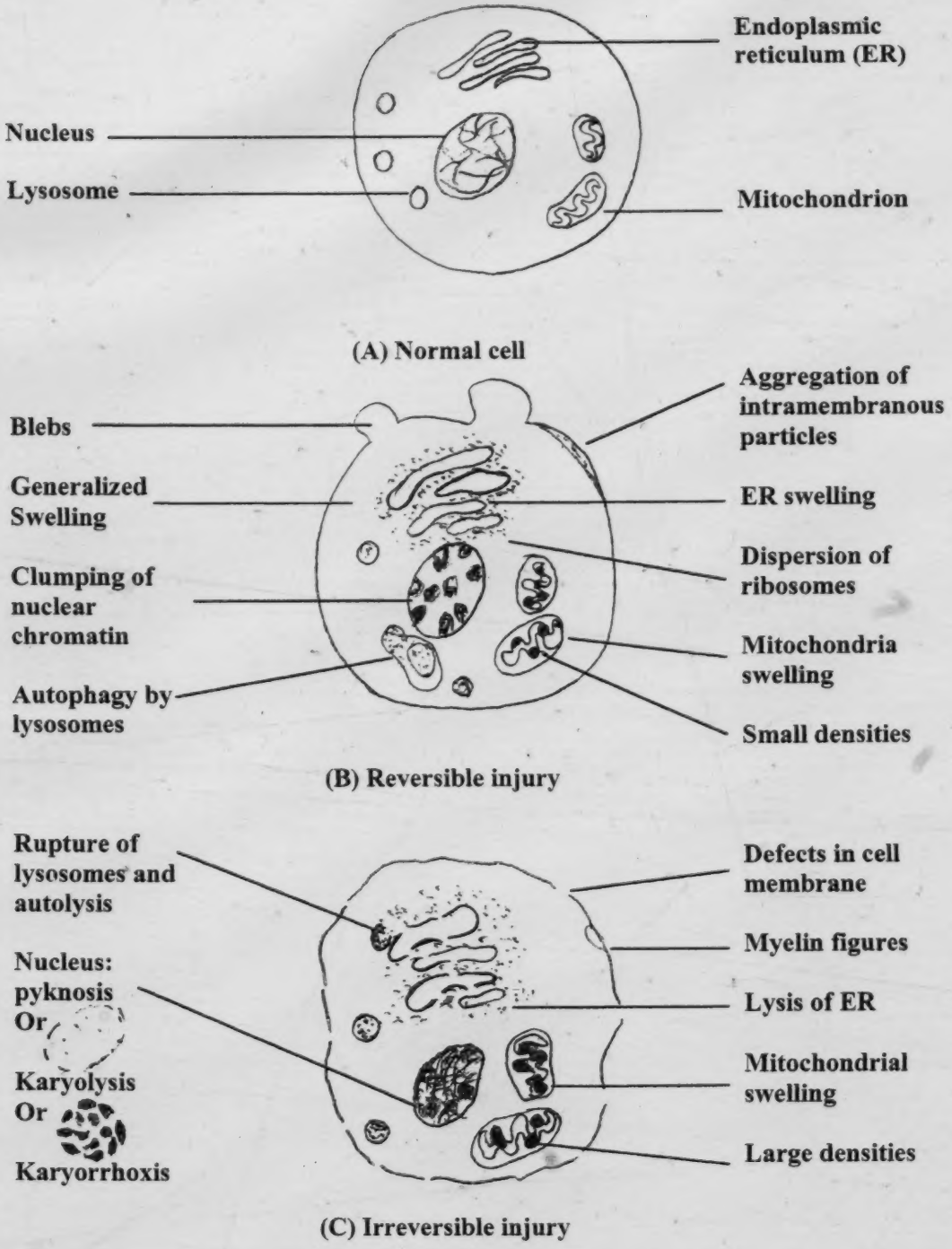

Figure 2-2. Cellular Injury and Cell Death (after Wyllie 1994)

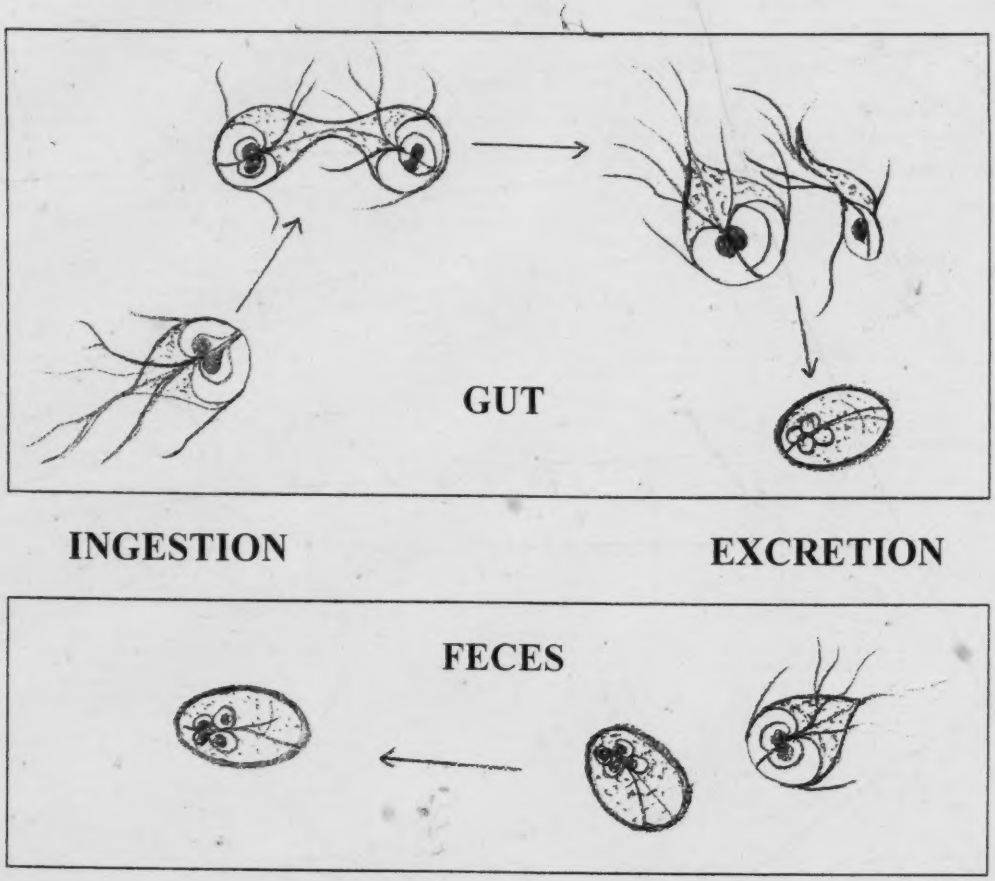

Figure 2-1. Life cycle of *Giardia* spp. (adapted from Meyer and Jarroll, 1980)

3. MATERIALS AND METHODS

3.1 *Giardia* Methods

3.1.1 *In vitro* Cultivation of *Giardia lamblia* Trophozoites

Trophozoites of WB strains of *G. lamblia* were maintained in axenic culture by subculture twice weekly in Diamond's TYI-S-33 medium (Diamond *et al.* 1978). Cultures were grown to confluence at 37 °C in polystyrene culture flasks.

Trophozoite growth medium was a filter sterilized TYI-S-33 medium containing 20 mg/L trypticase peptone (BBL-Hyclone), 10 mg/L yeast extract (BBL), 10 mg/L dextrose (Sigma), 2 mg/L NaCl (BDH), 2 mg/L L-cysteine HCl (BDH), 0.2 mg/L ascorbic acid (BDH), 1 mg/L dipotassium orthophosphate, 0.6 mg/L potassium dihydrogen orthophosphate, 0.02 mg/L ferric ammonium citrate (Sigma), and 0.25 mg/L bacteriological grade bovine bile (Sigma). After adjusting the pH to 7.1, the medium was filter sterilized using 0.2 μm Milli-pore filters. Then heat inactivated bovine calf serum (Hyclone) was added to make the final concentration 10%. The sterilized media was supplemented with gentamycin (Gibco). The growth medium was used immediately or stored at 4 °C for less than 1 week prior to use.

3.1.2 *In vitro* Encystation of *Giardia lamblia* Trophozoites

In vitro encystation was carried out following procedure developed by Kane (1991). After trophozoites were grown to confluence, the flasks were inverted several times, and the spent growth medium was poured off together with unattached trophozoites. The flasks were then filled with encystation medium, which consisted of growth medium adjusted to pH 7.8, containing bacteriological grade 10 mg/mL bovine bile and 0.5 mM lactic acid. Flasks were returned to 37 °C for 24 hours. Cultures were chilled and then pelleted by centrifugation at 400x*g* for 10 min. Encystation medium

were poured off, replaced with freshly prepared growth medium. After incubation in growth medium for 24 hours, cultures were harvested by chilling the flasks, followed by centrifugation at 400x *g* for 10 min. Non-encysted trophozoites were eliminated by hypotonic lysis in distilled water for 1 hour at 4 °C.

3.1.3 Purification of *Giardia lamblia* Cysts

The purification of viable cysts from non-viable cysts and trophozoites was done using cesium chloride gradient ultracentrifugation (Taghi-Kilani and Sekla 1987). A cesium chloride gradient was prepared in a 5 mL Beckman polyallomer ultracentrifuge tube. The placement consisted of a bottom layer (1 mL of 1.4 g/mL CsCl), middle layer (2 mL 1.1g/mL CsCl) and a top layer (1 mL of 1.05 g/mL CsCl). Approximately 500 mL of the parasite material was gently layered on top of the CsCl gradient and centrifuged 11000x*g* for 10 min using a swinging-bucket rotor (SW-55) at the slow brake setting (Beckman L7-55 ultracentrifuge). After centrifugation, the band interfacing the top layer (1.05 g/mL CsCl) and middle layer (1.1 g/mL CsCl) was removed and placed in 15 mL conical centrifuge tubes. The tubes were filled with deionized water and the cysts washed twice by centrifugation at 400 x g for 10 min. After the final washing step, the cysts were suspended in phosphate buffered saline. Cysts isolated by this procedure were free of debris, bacteria, and trophozoites. Viability of cysts as determined by the nucleic acid staining method was greater than 90%. Cysts were used within 3 days of isolation in all experiments. The cyst concentration in the suspension was determined by triplicate counts using a hemocytometer.

3.1.4 Viability Assessment Using Nuclei Acid Staining of *Giardia lamblia* Cysts

The fluorescent nucleic acid binding dye SYTO-9 has been previously tested (Taghi-Kilani *et al.* 1996) to determine the viability of *Giardia* spp. cysts. A live/dead BacLight ™ was found to the better of the two double staining viability kits tested.

After disinfection, the contents of the reactor were emptied into centrifuge tubes, rinsed with deioinized water, and centrifuged at 440x*g* for 10 minutes to pellet parasites. The supernatant was removed by aspiration leaving approximately 2 mL of concentrated sample containing the cysts. Cysts were counted 3 times using a hemocytometer before removing approximately 1 x 10^5 cysts for determination of viability using the nuclei acid binding dye. In trials where recovery was poor, fewer cysts were used. The remaining parasites were used for electron microscopy examinations. Cysts were suspended in test water (100 µL) and 10 µM of each reagent A and B of Baclight™ were added to the reaction tubes. After mixing, the tubes were wrapped in aluminum foil and incubated at 37 °C for 15 minutes. Cysts were visualized using a Nikon epi-fluorescence microscope with a B-1A filter combination of excitation at 490 nm and emission 520 nm. Cysts displaying yellow or orange staining were considered dead, whereas those showing green staining were considered viable.

3.2 Disinfection Methods

3.2.1 Oxidants and Apparatus

3.2.1.1 Ozone

Ozone gas was generated using a water-cooled corona discharge generator (Model T-816, Welsbach Ozone Systems Corporation, Sunnyvale, CA) from extra dry oxygen feed gas. Oxygen carrier gas containing approximately 5 percent ozone was bubbled

for a minimum of 20 minutes as 20 °C through 400 mL of deionized water pre-cooled to 4 °C in a 500 mL gas absorption flask. Ozone concentration in the stock solution was approximately 40 mg/L. The ozone residual in the reactor was neutralized using sodium formate.

Ozone residuals were determined by UV absorbance at 260 nm. A molar absorptivity of 3,300 l/(M·cm) was used (Hart *et al.* 1983).

3.2.1.2 Chlorine

Free chlorine stock solution was prepared weekly as needed using sodium hypochlorite solution (6 percent available chlorine, BDH Inc., Poole, England) with oxidant demand-free water to give a concentration of about 300 mg/L. Chlorine concentration was determined by the DPD procedure (Greenberg *et al.* 1992). The stock chlorine solution was stored in dark refrigerated conditions during the day of the experiment. Chlorine residual in the reactor after treatment was neutralized using sodium sulfite.

3.2.2 Oxidant Demand-Free Material

3.2.2.1 Oxidant Demand-Free Glassware

All glassware was initially cleaned using a detergent specifically designed for laboratory glassware and washed in deionized distilled water with a 20 percent phosphate acid rinse followed by two more high temperature rinses with deionized water. After the initial cleaning, all glassware was rinsed three times with deionized water including one more phosphate acid rinse. From then on, all glassware was cleaned using hot deionized water.

The disinfection reactor, stir bar, pipette tips and any other glassware that may come in contact with the test solution were made oxidant demand-free to reduce the oxidant

demand. All glassware was made oxidant demand free by completely filling the containers with water containing 15 to 20 mg/L of dissolved ozone and then capping them with aluminum foil. After a minimum contact time of 30 minutes, the water was poured out, the foil was replaced, and the containers were placed in an oven to dry.

3.2.2.2 Oxidant demand-free water and phosphate buffer

Deionized water obtained from an Elga® system (ELGASTAT MAXIMA-HPLC, Elga Ltd. England) operated at a resistivity of at least 18 M Ω/cm was used. The standard 0.05 M phosphate buffer was prepared using potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate (BDH Inc. AnalaR Grade, England) adjusted to the desired pH of the experiments with a strong acid (0.1 M hydrochloric acid) or a strong base (0.1 M sodium hydroxide). The resulting solution was made oxidant demand-free by bubbling ozone through the solution for 40 minutes, followed by boiling for 20 minutes and cooling in a clean air hood. The pH of the phosphate buffer was measured (Accumet Model 25 pH/ion meter, Fisher Scientific) when it was cooled down to room temperature. This procedure was repeated until the measured pH was $\pm$ 0.1 of the target pH value. The resulting solution was found to be ozone and chlorine demand-free. The buffered solution was found to resist pH drift adequately under all oxidant dosing conditions. The oxidant demand-free water and buffer solution were stored in 4 L brown reagent bottles at room temperature and were used within 3 months.

3.2.2.3 Reactor Vessels

The reactor vessels used were oxidant demand-free 250 Erlenmeyer flasks. A diode-array spectrophotometer (Hewlett-Packard Model 8452A) with a 10 mm light path and 35 µL flow through cell was operated in a closed loop to continuously monitor ozone residual as described elsewhere (Finch *et al.* 1992).

3.2.3 Temperature Control

Temperature in the reactor was controlled by an orbital shaking water bath with microprocessor control (Model 3545, LAB-LINE INSTRUMENTS Inc., Illinois) that maintained a stable temperature within $\pm$ 0.1 °C. The water bath was chilled using an off-line refrigerator. The reactor was partially submerged in the water bath.

3.2.4 Determination of Oxidants Concentrations

3.2.4.1 Ozone

The concentration of ozone aqueous stock solution was measured by ultraviolet spectrophotometry (10 mm cuvette, Ultraspec 2000, UV/Visible spectrophotometer, Pharmacia Biotech Ltd., Cambridge, England) at 260 nm with the molar absorptivity of 3,300 l/M·cm). Hart *et al.* (1983) indicated that the molar absorption efficient of ozone in pure water is 3292 $\pm$ 70 l/M·cm). Due to the quick decay in ozone stock solution (half-life about 20 minutes for aqueous ozone at neutral pH in pure water and room temperature), the ozone stock solution was freshly prepared before each experiment. Before adding ozone stock solution to the reactor, aeration by the ozone product gas was stopped. Three measurements of ozone concentration in the stock solution were taken before applying it to the reactor. Three more measurements were taken right after applying ozone. The mean of these six measurements was considered the concentration of ozone stock solution in calculation of the applied dose.

The real time ozone concentration was also monitored by the ultraviolet spectrophotometry (HP 8452A Diode Array spectrophotometer, Hewlett-Packard Company) at 260 nm. The spectrophotometer sample pump continuously sampled the reactor contents using a closed loop at a flow rate of 8 mL/min. The cuvette

holder of HP 8452A spectrophotometer was cooled by the circulating cooling water to prevent the fog forming on the cuvette surface at low temperature.

3.2.4.2 Chlorine

The concentration of chlorine stock solutions was determined by the DPD colorimetric procedure (Greenberg *et al.* 1992). Free chlorine powder pillows (HACH company, Coveland, CO) for 25 mL sample volume were used in the measurement. A standard curve was prepared monthly using potassium permanganate solution prepared by dissolving 891 mg $KMnO_4$ (BDH AnalaR reagent grade, dried at 105 °C for 1 h) into 1,000 mL oxidant demand-free water. This 1,000 mg/L stock solution was diluted ten-fold to 100 mg/L with oxidant demand-free water in a volumetric flask. One milliliter of this solution was diluted to 100 mL with oxidant demand-free water, to produce a chlorine equivalent of 1.00 mg/L in the DPD reaction. A serial dilution of $KMnO_4$ standards was prepared to cover the chlorine equivalent range of 0.05 to 4 mg/L. A DPD powder pillow was added to each $KMnO_4$ standard solution, and then its absorbance at 515 nm (Ultraspec 2000, UV/Visible spectrophotometer, Pharmacia Biotech Ltd., Cambridge, England) was measured immediately. A standard curve was developed based on response of the absorbance versus equivalent free chlorine concentration.

The concentration of free chlorine in the reactor was measured using the free chlorine pillows. The free chlorine was usually within the range of the standard curves. If not, the samples were diluted before re-measurement. Twenty five milliliters of sample was put into a 50 mL oxidant demand-free Fisher brand borosilicate glass vial, and a package of DPD powder was added to the mixture. The absorbance was measured at 515 nm. In the disinfection experiment, usually 5 mL of sample was taken from the reactor using an ODF oxford pipette and diluted 5 folds for measurement of free chlorine concentration in the reactor.

3.2.5 Microorganism Inactivation Procedure

Experiments were conducted in oxidant demand-free water using batch reactors containing between 5 x 10^5 and 2 x 10^6 *G. lamblia* cysts. The reactors were wrapped in aluminum foil and covered with a foil lip to minimize the volatilization and photodecomposition of the oxidants, and mixed in the orbital shaking water bath. The reactors, test water and cysts were brought to the desired temperature slowly using the temperature controlled water bath. The cysts were acclimated to the water bath for at least 60 minutes prior to addition of oxidants.

A control reactor containing cysts was run side by side with the experimental series conducted for each trial day. The control was processed exactly the same as the experimental trial except for the addition of the oxidant.

3.2.5.1 Single Oxidants

Experimental protocols for treatment followed a modified procedure described elsewhere (Finch *et al.* 1994). Typically, the cysts were suspended in a 100 mL of oxidant demand-free 0.05 M phosphate buffer solution in a 250 Erlenmeyer flask. Oxidant was applied to the reactor by adding a measured volume of oxidant working stock solution using a calibrated pipette. The residual disinfectant concentration in the reactor was monitored continuously by ultraviolet spectrophotometry (HP 8452 Diode Array Spectrophotometer, Hewlett-Packard Company) as described earlier in section 3.2.2.3. At the end of the contact time, the residual disinfectant was neutralized by quenching reagents (1 M sodium formate for ozone, 0.05 M sodium sulfite for free chlorine). Cyst samples after treatment were stored in the 50 mL conical centrifuge tubes at 4 °C. Viability assessment was done on the treated cysts within 12 hours after disinfection procedure.

3.2.5.2 Sequential Oxidants

The procedures for preparing test water and cysts were similar to those used for a single disinfectant (Figure 3-1). Typically, 5×10^5 and 2×10^6 *G. lamblia* cysts were added to 100 mL buffer in a 250 mL Erlenmeyer flask. For experiments with ozone treatment followed by free chlorine, the real time ozone concentration in the reactor was monitored by HP 8452A diode array spectrophotometer. At the end of ozone contact time, ozone residual was removed by adding sodium formate. A sample containing ozone treated cysts was taken from the reactor. Immediately, free chlorine was applied by adding a measured volume of free chlorine stock solution. The concentration of free chlorine residual in the reactor was measured by the DPD procedures (Greenberg *et al.* 1992). One or two more samples were taken from the reactor to measure gross kill at different contact times. The residual free chlorine was neutralized by 0.05 M sodium sulfite.

For experiments with free chlorine followed by ozone, ozone was added immediately at the end of the chlorine contact time without quenching of the free chlorine residual (Figure 3-1). At the end of ozone application (typically 1 min or 5 min), 0.05 M sodium sulfite and 1 M sodium formate were added to quench both the ozone and free chlorine residual. Inactivation by the second disinfectant alone was measured separately following the procedures used for single treatment.

The synergy of the sequential disinfection was calculated as

$$\text{Synergy} = I_r - (I_{r1} + I_{r2})$$

Where I_r = gross logistic inactivation of sequential disinfection measured in the experiment (log-units);

I_{r1} = logistic inactivation by first disinfectant measured experimentally (log-units);

I_{r2} = logistic inactivation by second disinfectant under the same condition and contact time as was used in the second treatment (log-units)

3.2.6 Experimental Design

The concentration and time conditions used for each combination of experiments were chosen to examine varying degrees of cell injury, while maintaining an inactivation within the limits of detection. Single chemical experiments were carried out at 25 °C and 5 °C. Multiple chemical experiments were carried out at 5 °C in order to maintain the gross inactivation level within 2 log-units. All experiments were carried out at pH 6. Each concentration and contact time combination was tested at least twice as an individual trial.

For multiple chemical treatments, 2 levels of pre-treatment kill were investigated, low and high. The inactivation level between 0.2 to 0.4 log-units was targeted for low level pre-treatment and between 0.8 to 1.2 log-units was targeted for high level pre-treatment.

3.3 Transmission Electron Microscopy Methods

Untreated and treated cysts were washed two times in deionized water and the centrifuged pellet was pre-fixed with 2.5% glutarldehyde in Millonig's buffer (pH 7.2) at room temperature for one hour. The specimen was then washed in the same buffer three times for 10 minutes each. Then the specimen was post-fixed with 1% osmium tetroxide (OsO_4) in the same buffer at room temperature for one hour. After washing in distilled water briefly, the specimen was dehydrated in a series of ethanol (50% to 100%) for 10 minutes each. Thin sections were cut on an LKB Ultrotome, stained in uranyl acetate and lead citrate and viewed and photographed with a HITACHI H600 Transmission Electron Microscope.

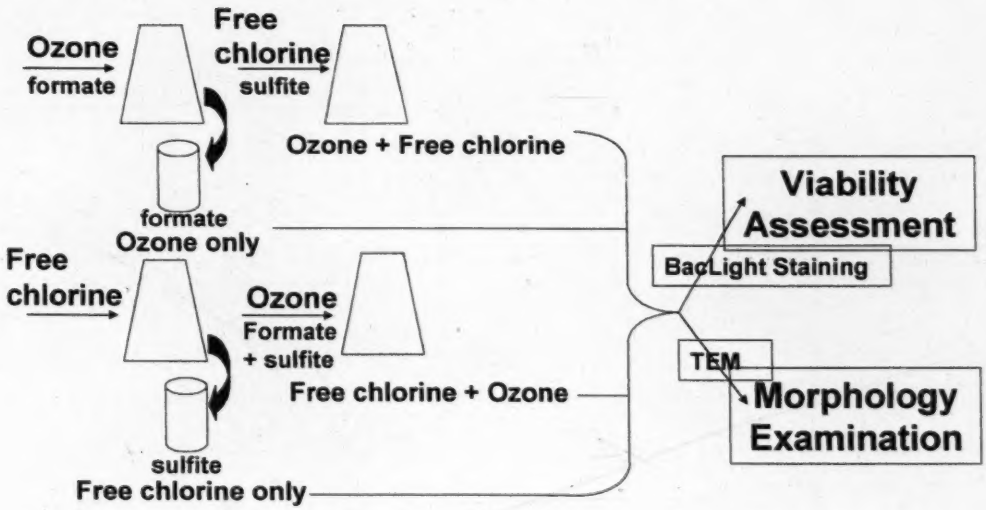

Figure 3-1. Experimental procedure for the sequential treatment of *Giardia lamblia* cysts with ozone and free chlorine

4. RESULTS AND DISCUSSION

4.1 Microbial Inactivation of *Giardia lamblia*

4.1.1 Single chemicals

4.1.1.1 Ozone

Experimental data for ozone inactivation at 25 °C and 5 °C and pH 6 are summarized in Table 4-1 and Table 4-2. Three levels of inactivation, namely low, moderate and high (approximately 0.5, 1, and 2 log-units respectively) were targeted for subsequent morphological studies.

Table 4-1. *G. lamblia* cyst inactivation by ozone in pH 6 phosphate buffer at 25 °C

Target Inactivation Level	n	Concentration (mg/L)	Contact time (min)	CT (mg·min/L)	Observed Inactivation (log-units)
Low	2	0.1	0.5	0.05	0.3 to 0.4
Moderate	2	0.5	2	1.0	1.2 to 1.4
High	2	0.5	5	2.5	1.9 to >2.0

Table 4-2. *G. lamblia* cyst inactivation by ozone in pH 6 phosphate buffer at 5 °C

Target Inactivation Level	n	Concentration (mg/L)	Contact time (min)	CT (mg·min/L)	Observed Inactivation (log-units)
Low	5	0.2	1	0.2	0.3 to 0.4
Moderate	3	0.5	5	2.5	1.2 to 1.5

At 25 °C, the ozone doses ranged from 0.1 to 0.5 mg/L and the contact time 0.5 to 5 minutes, with the observed kill from 0.3 to greater than 2.0 log-units. Due to poor recovery of cysts following treatment, the viability measurement limit was below 2 log-units in many trials. Single chemical treatments were also conducted at lower temperature to maintain gross inactivation levels to less than 2 log-units in sequential treatment experiments. Experimental data for ozone inactivation level at 5 °C are summarized in Table 4-2. At 5 °C, the doses and contact times required for the same levels (low and moderate) of inactivation were much higher than those at 25 °C. Ozone concentration of 0.2 mg/L and 1 minute contact time resulted in 0.3 to 0.4 log-units of inactivation. The CT required is approximately 4 times that required for the same level of inactivation at 25 °C. An ozone concentration of 0.5 mg/L and 5 minute contact time resulted in 1.2 to 1.5 log-units of inactivation. The CT required was approximately 2.5 times that required for the same level of inactivation at 25 °C.

4.1.1.2 Free Chlorine

Experimental data for free chlorine inactivation at 25°C and 5°C and pH 6 are summarized in Table 4-3 and Table 4-4. Three levels of inactivation, namely low, moderate and high (approximately 0.5, 1, and 2 log-units respectively) were targeted for subsequent morphological studies. At 25 °C, the free chlorine doses ranged from 1.8 to 5.0 mg/L and the contact time 30 to 120 minutes, with the observed kill from 0.4 to over 1.7 log-units. Experimental data for free chlorine inactivation level at 5 °C is summarized in Table 4-4. At 5 °C, the doses and contact times required for the same levels (low and moderate) of inactivation were higher than those at 25 °C. Free chlorine concentration of 1.9 to 4.9 mg/L resulted in 0.2 to 0.8 log-units of inactivation. The same concentration and contact times at 25 °C resulted in approximately twice the inactivation levels.

Table 4-3. *G. lamblia* cyst inactivation by free chlorine in pH 6 phosphate buffer at 25 °C

Target Inactivation Level	n	Concentration (mg/L)	Contact time (min)	CT (mg·min/L)	Observed Inactivation (log-units)
Low	2	1.8 to 2.0	30	54 to 60	0.4 to 0.5
Moderate	2	1.8 to 2.0	100	180 to 200	0.9 to 1.2
High	2	4.8 to 5.0	120	560 to 600	1.6 to 1.7

Table 4-4. *G. lamblia* cyst inactivation by free chlorine in pH 6 phosphate buffer at 5 °C

Target Inactivation Level	n	Concentration (mg/L)	Contact time (min)	CT (mg·min/L)	Observed Inactivation (log-units)
Low	4	1.9 to 2.0	30	57 to 60	0.2 to 0.3
Moderate	3	4.5 to 4.9	120	542 to 586	0.6 to 0.8

4.1.1.3 Discussion of Single Chemical Treatment

A comparison of CT values presented by previous studies to CT values resulting from this study is summarized in Table 4-5 and Table 4-6. Results from this study show much higher CT values than previously reported. This may be attributed to the nature of the pathogen used as well as the method of viability measurement. Firstly, the *G. lamblia* cysts used in this study were derived *in vitro*. Differences may exist in the structure, biochemistry and metabolism of cysts derived *in vitro* compared to those isolated from animals. These differences may result in increased resistance to chemical inactivation. Secondly, the viability assay used may have significantly over-estimated ozone inactivation. Nucleic acid staining was based on the cyst wall permeability. Although studies have shown correlation between SYTO-9 and animal infectivity (Taghi-Kilani *et al.* 1996), recent studies showed that SYTO-9 predictive models must be developed on a chemical disinfectant-specific basis, and limited to a given set of experimental conditions, because changes in parasite cell wall permeability to the SYTO-9 dye are dependent on the type of oxidant used (Gyürék *et al.* 2001; Neumann *et al.* 2000).

The effect of temperature on the inactivation of *G. lamblia* cysts in this study agrees with previous studies (Jarroll *et al.* 1981b; Wickramanayake *et al.* 1984a). Comparison of CT products indicates that *G. lamblia* cyst resistance to ozone or free chlorine at 5 °C is approximately 2 times more than that at 25 °C. The U.S. Environmental Protection Agency suggests a factor of 2 be used for every 10 °C decrease in water temperature within the range of 1 to 25 °C. Wickramanayake (1984a) observed 3 times more resistant cysts to ozone at 5 °C than that at 25 °C. The same trend was observed in cysts treated with free chlorine (Jarroll *et al.* 1981a). Cyst resistance to chlorine increased by 1 order of magnitude as temperature dropped from 25 °C to 5 °C at pH 7.

Table 4-5. Summary of reported ozone treatment requirements for 99 percent inactivation of *Giardia lamblia* cysts

Species	Viability measurement method	Ozone protocol	Temperature (°C)	CT (mg·min/L)	Reference
G. lamblia	Nuclei acid staining	Batch liquid, batch ozone	25	2.5[1]	This study
G. lamblia	Excystation	Batch liquid, continuous gas	5	0.53[1]	(Wickramanayake *et al.* 1984a)
			25	0.17	
G. lamblia	Animal infectivity	Batch liquid, batch ozone	22	0.8[2]	(Finch *et al.* 1993b)

NOTE:

1. CT is calculated as Concentration x Contact time
2. CT is calculated as integrated residual concentration x Contact time

Table 4-6. Summary of reported chlorine treatment requirements for 99 percent inactivation of *Giardia lamblia* cysts

Species	Viability measurement method	pH	Temperature (°C)	CT (mg·min/L)	Reference
G. lamblia	Nuclei acid staining	6	25	600[1]	This study
G. lamblia	Excystation	6	5	75[1]	(Jarroll *et al.* 1981a)
G. lamblia	Excystation, Animal infectivity	7	5	120	USEPA
			25	32	

NOTE:

1. CT is calculated as Concentration x Contact time

4.1.2 Multiple chemicals

4.1.2.1 Ozone Followed by Free Chlorine

Data for ozone followed by free chlorine sequential inactivation of *G. lamblia* cysts are summarized in Table 4-7. The relationship between the gross inactivation level and the free chlorine CT product is illustrated in Figure 4-1. Two levels of ozone pre-treatment, the low and the moderate, were investigated. The low level ozone pre-

treatment used an ozone dose of 0.2 mg/L for 1 minute, resulting in 0.3 to 0.4 log-units of inactivation. For the second treatment, the free chlorine CT products ranged from 53 to 784 mg·min/L, resulting in a gross kill from 0.5 to greater than 2.0 log-units for the sequential treatment. About 0.1 to greater than 0.5 log-units of synergy was observed.

The moderate level ozone pre-treatment used a dose of 0.5 mg/L for 5 minutes, which resulted in 1.2 to 1.5 log-units inactivation. The free chlorine CT product for the second treatment ranged from 57 to 567 mg·min/L, resulting in a gross kill of greater than 2.0 log-units for the sequential treatment. Greater than 0.5 log-units of synergy were observed.

Table 4-7. Summary of *G. lamblia* cyst inactivation using ozone followed by free chlorine in oxidant demand-free 0.05 M phosphate buffer at pH 6 and 5 °C

First treatment (ozone)		Second treatment (free chlorine)		Total		
CT (mg·min/L)	Kill (log-units)	CT (mg·min/L)	Kill (log-units)	Additive kill (log-units)	Observed kill (log-units)	Synergy (log-units)
0.2	0.3	53	0.2	0.5	0.6	0.1
0.2	0.4	56	0.2	0.6	0.6	0
0.2	0.3	138	0.2	0.4	0.5	0.1
0.2	0.4	160	0.2	0.6	0.7	0.1
0.2	0.3	530	0.6	0.9	1.3	0.4
0.3	0.4	784	0.8	1.2	1.6	0.4
2.4	1.5	57	0.2	1.7	> 2.0	> 0.4
2.5	1.2	57	0.2	1.4	> 2.0	> 0.6
2.5	> 2.0	546	0.7	>2.7	> 2.0	?
2.4	1.3	567	0.8	2.1	> 2.0	?

From the inactivation curve (Figure 4-1) for ozone and free chlorine sequential inactivation, gross kill increased linearly with the free chlorine CT product. The

slope of the inactivation curve of low ozone pre-treatment followed by free chlorine (0.0016 log-units·L·min/mg) was steeper than that of free chlorine treatment alone (0.0009 log-units·L·min/mg).

4.1.2.2 Free Chlorine Followed by Ozone

Data for free chlorine followed by ozone sequential inactivation of *G. lamblia* cysts are summarized in Table 4-8. The relationship of the gross inactivation level to the ozone CT product was illustrated in Figure 4-2. Two levels of free chlorine pre-treatment, the low and the moderate, were investigated. The low level free chlorine pre-treatment used free chlorine dose of 2 mg/L and 5 mg/L for 30 minutes, resulting in 0.2 log-units of inactivation. For the second treatment, the ozone CT products ranged from 0.2 to 2.3 mg·min/L, resulting in a gross kill from 0.9 to greater than 2.0 log-units for the sequential treatment. About 0.3 to greater than 0.8 log-units of synergy was observed.

The moderate level free chlorine pre-treatment, using a free chlorine CT product of 528 to 76 mg·min/L, resulted in 0.6 to 0.8 log-units inactivation. The ozone CT product for the second treatment ranged from 0.2 to 2.3 mg·min/L, resulting in a gross kill of greater than 2.0 log-units for the sequential treatment. Greater than 1.1 log-units of synergy were observed.

Table 4-8. Summary of *G. lamblia* cyst inactivation using free chlorine followed by ozone in oxidant demand-free 0.05 M phosphate buffer at pH 6 and 5 °C

First treatment (chlorine)		Second treatment (ozone)		Total		
CT (mg·min/L)	Kill (log-units)	CT (mg·min/L)	Kill (log-units)	Additive kill (log-units)	Observed Kill (log-units)	Synergy (log-units)
56	0.2	0.2	0.3	0.6	0.9	0.3
138	0.2	0.2	0.3	0.4	1.1	0.7
58	0.2	2.2	1.5	1.7	> 2.0	> 0.2
58	0.2	2.3	1.2	1.5	2.3	0.8
530	0.6	0.2	0.3	0.9	> 2.0	> 1.1
776	0.8	0.2	0.4	1.2	> 2.0	> 0.8
558	0.8	2.3	1.3	2.1	> 2.0	?
528	0.7	2.3	> 2.0	> 2.0	> 2.7	?

4.1.2.3 Discussion of Multiple Chemical Treatment

Sequential treatment of *G. lamblia* cysts using ozone followed by free chlorine showed synergy, especially with higher second treatment levels. Many studies have shown that treatment of protozoan cysts with ozone followed by free chlorine show significant synergy (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c). Bradbury (1998) reported significant synergy in *G. muris* cysts treated with ozone followed by free chlorine at pH 6 and 8.

When the treatment sequence was reversed, the gross kill was observed to be much higher. Greatest synergy was observed in treatment of *G. lamblia* cysts with free chlorine followed by ozone. Although this particular sequence has not been previously studied, greater synergy was observed by Li (2001b) when free chlorine

was used as the second chemical following ozone pre-treatment than monochloramine. The author suggested that the extent of sub-lethal injury that could cause synergy depended on the oxidant used in the second treatment. A stronger oxidant in the second treatment requires a relative minor injury for synergy. In this study, ozone was a much stronger oxidant than free chlorine. Pre-conditioning of *G. lamblia* cysts for subsequent ozone treatment, resulted in greater synergistic effects than for subsequent free chlorine treatment.

4.2 Morphology of *Giardia lamblia* Cysts

4.2.1 Untreated *Giardia lamblia* Cysts

The transmission electron micrographs of control *G. lamblia* cysts are shown in Figures 4-3, 4-4 and 4-5. Control *G. lamblia* cysts were processed in the same way as cysts of the treated group except for the addition of the oxidant. Processing conditions, including the application of quenching agents 1M sodium formate and 0.05 M sodium sulfite, did not appear to have an effect on the morphology of cysts. The control cysts were enclosed within walls that consisted of thin fibrous elements. The cyst wall had a uniform thickness of approximately 0.3 μm and was closely associated with or attached to the plasma membrane of the parasite (Figures 4-3, 4-4 and 4-5). An electron micrograph with higher magnifications (Figure 4-3B) shows a thin cytoplasm layer between the membrane underlying the cyst wall and the membrane surrounding the parasite. There was extensive contact between the main cytoplasmic mass and the cytoplasmic mass underlying the cyst.

The lacunar space located peripherally contained materials of very low electron density (Figure 4-3B). The size of the lacunar space varied based on the section observed. A large number of circular to oval vacuoles with low electron density were found just beneath the cell membrane of the parasite (Figures 4-3, 4-4 and 4-5). These peripheral vacuoles were enclosed within a membrane.

The nucleus was spherical to oval in shape and was surrounded by a nuclear envelope (Figure 4-4A). The nucleoplasm was uniform in density; nucleoli had not been observed. Nuclei of some of cysts were elongated and had different configurations (Figure 4-4B). In such cases, not more than two nuclei were observed in a cyst.

The flagellar axonemes were found in the area of the nuclei (Figure 4-4). In lateral sections, a maximum of 8 axonemes was observed in a single cyst. When 8

axonemes were present 6 were located in the central area near the nuclei with 2 at the periphery. The free flagella were enclosed by the plasma membrane and were found in the lacunar space (Figure 4-3B). Each axoneme consisted of two single microtubules surrounded by 9 pairs of microtubules.

The adhesive disk was disassembled and appeared as stacks or crescents throughout the cyst cytoplasm (Figure 4-4 and 4-5).

Other cytoplasmic organelles such as mitochondria, Golgi vesicles and the endoplasmic reticulum were not observed in the sections in the present study.

4.2.2 Single Chemical Treated *Giardia lamblia* Cysts

4.2.2.1 Ozone

4.2.2.1.1 Low Ozone Treatment

A transmission electron micrograph of *G. lamblia* cysts treated with 0.1 mg/L of ozone for 30 seconds at 25 °C and pH 6 are shown in Figure 4-6A. Under these conditions the inactivation level was 0.3 to 0.4 log-units (Table 4-4). The closely attached fibrous elements of the cyst wall became dispersed causing the cyst wall thickness to increase. The two membrane layers of the cyst wall, although still intact, had become irregular and appeared ruffled. In most cysts, membrane bound peripheral vacuoles underlying the cyst wall were uniformly sized and shaped. Electron density was relatively low for most vacuoles. The nucleus still maintained its round to oval shape, with the nuclear envelope still intact. No alterations were seen in the internal components such as striated adhesive disk fragments and flagellar axonemes.

Cysts treated with 0.2 mg/L of ozone for 1 min at pH 6 and 5 °C (0.3 to 0.4 log-units inactivation) showed damage similar to that discussed above (Figure 4-6B). During

this short ozone contact period at low temperature, mostly the cyst wall and sections of the plasma membrane were affected.

4.2.2.1.2 Moderate Ozone Treatment

Extensive damage was noted in Figures 4-7, 4-8 and 4-9, after exposure to 0.5 mg/L of ozone for 2 minutes at pH 6 and 25 °C. These conditions, defined as moderate ozone treatment, resulted in inactivation levels between 1.2 to 1.4 log-units (Table 4-4). The cyst wall was altered showing noticeable irregularities, especially at the outer surface. The fibers of the cyst wall had loosened significantly. The plasma membrane underlying the cyst wall and surrounding the organism was broken at several locations (Figures 4-7C and Figure 4-8B). The overall cyst shape did not change. The disruption of membranes might have permitted the material in the lacunae to diffuse into the cytoplasm and vice versa (Figure 4-8B). As a result, the lacunar space was enlarged (Figures 4-7 and 4-8). The parasites appeared to be detached from their protective cyst wall. Peripheral vacuoles of most cysts of this treatment group appeared normal with their intact membranes (Figures 4-7, 4-8 and 4-9A). In some cysts, as represented by Figure 4-9B, many vacuoles became disorganized or taken over by larger and irregularly shaped vacuoles. The membranes of some vacuoles were fused together.

More internal organelle disorganization took place. The cytoplasm showed electron lucent areas indicating dissolution of cytoplasmic organelles. Electron density around the nuclear periphery increased (Figure 4-7A), probably representing aggregates of condensed nuclear material. The nuclear membrane appeared to be damaged as the membrane had lost the sharp and continuous line seen in normal cysts.

Other internal organelles such as flagellar axonemes and striated adhesive disc fragments showed sharp details and appeared unaffected.

4.2.2.1.3 High Ozone Treatment

Transmission electron micrographs of *G. lamblia* cysts treated with 0.5 mg/L of ozone for 5 minutes at pH 6 and 25 °C are given in Figures 4-10 and 4-11. In this treatment group, approximately 2 log-units of cysts were inactivated. The damages noted in cysts exposed to moderate ozone were intensified in cysts of this treatment group. The plasma membranes in the cysts in these figures were broken; in some cysts, they were no longer visible, but electron dense granular deposits could be seen along the inner cyst walls (Figure 4-11B). These deposits might be remnants of the plasma membrane. Some circular to oval structures with low electron densities could be seen along the periphery. They resemble the vacuoles but lack the characteristic surrounding unit membrane (Figures 4-10 and 4-11B). Ozonation had apparently destroyed the membrane. The cytoplasm of a majority of cysts was closely applied to the cyst wall. Detachment of the parasite from the cyst wall, as seen in the moderate treatment group, was not observed.

One prominent feature noted in many cysts of this treatment group was the appearance of a large number of internal vacuoles in the cytoplasm (Figures 4-10A and 4-11A). Some of these internal vacuoles had partial membranes, some appeared as "whorls", and some were of irregular shapes.

In most cysts, the nucleus was unaffected. In a few cysts, the nuclear envelope appeared to have disintegrated (Figure 4-11B). The electron density of the nuclear material had decreased considerably. The axonemes and fragments of the striated disk appeared to be unaffected.

4.2.2.2 Free Chlorine

4.2.2.2.1 Low Free Chlorine Treatment

Transmission electron micrographs of *G. lamblia* cysts treated with 2.0 mg/L free chlorine for 30 minutes at pH 6 and 25 °C are given in Figures 4-12 and 4-13. Under

low free chlorine treatment conditions, 0.4 to 0.5 log-units of cysts were inactivated. Morphological changes that had occurred were similar to those in the low ozone treatment group.

The closely associated filamentous material of the cyst wall had loosened and resulted in a swollen cyst wall. Along with the altered cyst wall, the plasma membrane surrounding the cytoplasm was broken at several points (Figure 4-13B). Where there were noticeable damages to the membrane layers of the cyst wall, there also existed an increase in the size of the peritrophic space underneath the wall (Figure 4-13); in some cysts, enlargement of sections of peritrophic space along injured cyst wall created blebs (Figure 4-12B). Peripheral vacuoles underlying the cyst wall were still intact.

The nuclear membrane maintained its smooth and continuous outline. Electron density along the periphery of the nucleus had increased (Figure 4-12A). Inside the cytoplasm, internal structures such as flagellar axonemes and fragmented adhesive disc were clearly visible and were orderly situated.

4.2.2.2.2 Moderate Free Chlorine Treatment

Upon exposure to 2 mg/L free chlorine for 100 minutes at 25 °C and pH 6, 0.9 to 1.2 log-units of *G. lamblia* cysts were inactivated. Transmission electron micrographs are given in Figures 4-14 and 4-15. The cyst wall showed increasing irregularities. The filamentous layer of the cyst wall showed further increase in thickness. The membranous layers of the cyst wall had been broken on many points. The peritrophic space continued to enlarge in size. Membrane bound peripheral vacuoles were still abundant in some cysts (Figure 4-14).

Extensive cytoplasmic damage had taken place. A majority of cysts showed spongy or honeycombed cytoplasm (Figures 4-14 and 4-15) indicating granulation or aggregation of cytoplasmic material. The nuclei of cysts treated with a moderate free

chlorine dose showed a variety of ultrastructural changes. The nuclei of some cysts were still intact (Figure 4-15). In many cysts, the nuclei were severely damaged, being very irregular in outline. Part of the nuclear membrane and the nuclear material were missing (Figure 4-14B). In some cysts, only small dark masses were noticed in the granulated cytoplasm, suggesting nuclear structure (Figure 14A). No significant physical changes could be seen in axonemes and in the striated adhesive disk.

4.2.2.2.3 High Free Chlorine Treatment

G. lamblia cysts treated with 5 mg/L free chlorine for 120 minutes resulted in 1.6 to 1.7 log-units inactivation (Figure 4-16). The cyst wall had swollen significantly under these conditions. The membranous layers of the cyst wall were no longer visible in most cysts, but electron dense deposits can be seen along the inner cyst walls. The cytoplasm of the parasite applied closely to the cyst wall, and was not shrunken away from the cyst wall as observed in cysts exposed to lower free chlorine doses. Circular to oval structures with low electron densities could be seen along the periphery. They resemble the peripheral vacuoles but lack the characteristic membrane. The axonemes and fragments of the striated disk failed to give complete ultrastructural details. However, these cellular components did not appear to be broken down into subunits.

4.2.2.3 Discussion of Single Chemical Treatment

Electron micrograph examinations of *G. lamblia* cysts treated with various levels of ozone or chlorine showed similar morphological changes. Chemical inactivation of *G. lamblia* cysts seemed to be a series of events. The first site of attack was the cyst wall. Ozone or free chlorine attacked the cyst wall and loosened the closely bound fibrous elements of the cyst wall as indicated by the increase of the cyst wall thickness. The loosening of filaments made the cyst wall possibly more permeable to ozone or free chlorine. The membrane underlying the cyst wall then became

damaged. Subsequent penetration of ozone or free chlorine damaged the second membrane, which was the membrane surrounding the parasite. Continued penetration of disinfectants then affected the nucleus and other cytoplasmic organelles.

Progressive morphological changes following chemical treatment followed the developmental stages of necrotic cell death described by Wyllie (1994). The results from this study indicated that low levels of ozone or free chlorine, while lethal, did not cause extensive visible damage to internal organelles of the cyst. Most of the visible damage occurred on the surface or the periphery of the cyst. At lower treatment levels, cyst injury was characterized by enlargement of the lacunae, a phenomenon not seen in cysts exposed to higher treatment levels. It is possible that at low oxidant levels or treatment, damaged cyst walls and membranes permitted the leakage of cytoplasmic material into the lacunar space. Leakage of cellular contents led to the eventual cyst death. Cyst injuries were at a structural level. It could be speculated that cell injury at this level was reversible. At higher treatment levels, cytoplasmic components of the parasite were closely applied to the cyst wall components. Increased permeability of the cyst wall by the chemical oxidants facilitated subsequent oxidation of cytoplasmic components by the chemicals. Cell injuries in this case would be at a molecular level, thus making cyst death irreversible. It can be suggested that structural damage to the plasma membrane, a primary lethal event, was reversible; whereas damage to nuclear material and other cytoplasmic metabolic activities, though secondary, was irreversible.

Although ozone and free chlorine induced significant morphological changes to the *G. lamblia* ultrastructure, there were noticeable differences. One unique feature following ozone treatment was the formation of internal vacuoles. This was not observed in chlorinated cysts. There are two possible hypotheses to explain this phenomenon. The first hypothesis could be further support for the injury via excystation pathway. During excystation, due to the large membrane surface area

needed for both the formation of the two trophozoites accompanying excystation as well as the re-organization of the disassembled adhesive disc, membranous "whorl" formation is a necessary event. Secondly, formation of internal vacuoles could be an important step in the natural death pathway. It is possible that such vacuoles were formed from the swelling of endomembrane systems including the endoplasmic reticulum. Although, the endoplasmic reticulum (ER) could not be identified in this study, it had been demonstrated to exist in *Giardia* spp. (Gillin *et al.* 1996). In a study of apoptosis in cysts cells of *Sarcocytsis muris* and *S. bovifelis*, it was suggested that the first stage of degradation of cyst involved ultrastructural changes primarily in rough ER, whose membranes form vacuoles, with various kinds of membranous and non-membranous materials inside (Radchenko and Beier 1995; Radchenko *et al.* 1995). This step parallels the "point of no return" step described by Ginn (1969). In higher organisms, the swelling of mitochondria and ER marked "the point of no return" to viability. If this were indeed the case, the commitment to death in ozone treated cysts was more prominent than that in chlorine treated cysts.

Another difference between ozone and free chlorine-induced morphological changes was the observed nuclear damage. In ozone treated *G. lamblia* cysts, damage to the nucleus was a late event. Even in cysts treated with high ozone levels, nuclei were unaffected in most cysts. Whereas in chlorine treated cysts, nuclei injury seemed to be an early event. Aggregation and condensation of nuclear material were seen in cysts treated with low levels of free chlorine. Free chlorine reactivity with nuclear material may be stronger when compared to that of ozone. Dennis *et al.* (1979) noted that chorine has an affinity for RNA at a pH of 5.6 to 9.9, which was greater than its affinity for protein. CMP and AMP consumed chlorine at low pH more actively than did GMP, with UMP being un-reactive. The authors (Dennis *et al.* 1979; Noss and Olivieri 1985) concluded that the inactivation of f2 bacteriophage by chlorine was due to damage of the nucleic acids. Preferential reaction between chlorine with RNA rather than with the capsid protein was also documented by Noss and Olivieri (1985) who studied chlorination of f2 bacteriophage. Ozone inactivation of virus, on the

other hand, was attributed to the inactivation of the protein coat (deMik and deGroot 1977, Sproul and Kim 1980; Herbold *et al.* 1989; Kim *et al.* 1980; Kim *et al.* 1984; Riesser *et al.* 1977; Shinriki *et al.* 1988; Sproul *et al.* 1982; Yoshizaki *et al.* 1988).

The third difference between ozone and free chlorine-induced damage was differences in cytoplasm morphology. Ozone treated cysts developed low electron dense sections in the cytoplasm, indicating dissolution of cytoplasmic material. Free chlorine treated cysts developed matrix densities of granular types inside the cytoplasm, suggesting aggregation of cytoplasmic material. This may reflect differences in the oxidant's reactivity with proteins. Sykes (1965) reported that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant.

Interestingly, there were striking similarities between morphological events following chemical inactivation and those of excystation. Studies of excystation have reported rupture of peripheral vacuoles and release of their contents, retraction of trophozoites from the cyst wall, a progressive enlargement of the peritrophic space, and the loss of tight arrangements of microfibrils of the cyst wall (Buchel *et al.* 1991b). The authors also observed a detachment of the outer cytoplasmic membrane of the cyst wall, followed by its breaking and formation into numerous small vesicles which then became lodged between the wall and the organism. Similar events were seen following cyst exposure to lower levels of ozone and free chlorine. Studies have reported that low levels of chlorine treatment enhanced excystation rates of *Giardia* spp. It is possible that chemical oxidants such as ozone and free chlorine elicited similar response in *Giardia* spp. cysts as factors that are known to induce excystation.

4.2.3 Multiple chemicals

4.2.3.1 Ozone Followed by Free Chlorine

4.2.3.1.1 Low Ozone Pre-treatment

Transmission electron micrographs of cysts treated with low ozone (0.2 mg/L for 1 min at pH 6 and 5 °C) followed by low chlorine (2 mg/L for 30 min at pH 6 and 5 °C) are shown in Figures 4-17, 4-18 and 4-19. Approximately 0.6 log-units of cysts were inactivated under these conditions.

Filaments of the cyst wall became loosely bound (Figures 4-17 and 4-18). In some cysts, there even appeared to be a loss of the cyst wall since the wall did not stain (Figure 4-19). Membranes underlying the cyst wall were grossly damaged, as only electron dense deposits could be seen along the wall (Figure 4-18B). In cysts which "lacked" a filamentous wall layer, the parasite seemed to be enclosed with only the plasma membrane which was also damaged (Figure 4-19). Membranes of the peripheral vacuoles appeared damaged or disintegrated (Figures 4-17, 4-18 and 4-19).

Cysts of this category lacked organized intracellular components. Internal vacuolization took place, as seen in cysts recovered from high ozone treatment group (Figure 4-17). The cytoplasm developed matrix densities as seen in cysts treated with free chlorine alone, suggesting aggregation of cytoplasmic material. The shape of nuclei in most cysts was no longer spherical or ovoid (Figure 4-17 and Figure 4-19). In cysts where the nuclei maintained its normal shape, electron density increased indicating condensation of the nuclear material (Figure 4-18). Flagellar axonemes and striated adhesive discs did not appear to be affected.

When the residual concentration and contact time of the second disinfectant, free chlorine, were increased to 5 mg/L for 120 min, significant synergy (0.4 log-units inactivation) was observed. Under these conditions, approximately 1.5 log-units

cysts were inactivated. Physical damages were similar to that discussed in cysts recovered from the low ozone followed by low free chlorine treatment group. All cysts showed spongy or honeycombed cytoplasms (Figure 4-20). Striated adhesive discs could no longer be detected in the sections cut. Flagellar axonemes no longer showed sharp details.

4.2.3.1.2 High Ozone Pre-treatment

Cysts treated with 0.5 mg/L ozone for 5 minutes at pH 6 and 5 °C followed by free chlorine resulted in over 2 log-units inactivation. In most cysts, there was a complete loss of recognizability of internal organelles (Figure 4-21A and 4-22). Even the most resistant organelles such as flagellar axonemes and striated adhesive disc lacked recognizability. The majority of cysts showed a spongy or honeycombed cytoplasm. Scattered, dark-stained spots were noticeable, suggesting remnants of nuclear material. In a few cysts (Figure 4-21B and Figure 4-22), nuclei were vague in outline, but were still noticeable.

4.2.3.2 Free Chlorine Followed by Ozone

4.2.3.2.1 Low Free Chlorine Pre-treatment

Cysts treated with free chlorine followed by ozone resulted in greater gross kill than those treated with the same levels of free chlorine and ozone in reverse sequence. Even with low free chlorine pre-treatment (2 mg/L for 30 minutes at pH 6 and 5 °C), significant synergy (0.3 log-units) were observed. The extent of physical damage as demonstrated in Figures 4-23, 4-24 and 4-25 was significantly greater than those of cysts treated with the same disinfectants in the reverse sequence. Some peripheral vacuoles were bound by partially intact membranes. Large sections in the cytoplasm were electron lucent and appeared as empty spaces (Figures 4-24B and 4-25A). Only the flagellar axonemes and fragments of the striated adhesive discs could be recognized. In a few cysts (Figure 4-23), nuclei could be seen. Electron dense

masses were present along the periphery of the nuclei, indicating aggregation of nuclear material.

4.2.3.2.2 High Free Chlorine Pre-treatment

Cysts pretreated with 5 mg/L free chlorine for 120 to 150 minutes at pH 6 and 5 °C are presented in Figures 4-26, 4-27, 4-28 and 4-29. Physical damages were similar to those seen in the low free chlorine pre-treatment group. The most prominent characteristic of cysts in this group was the extent of cytoplasmic dissolution as illustrated by the large sections of electron lucent spaces inside the cyst. However, even with the largest doses (Figure 4-29), the cysts maintained their overall round to ovoid shape. Fibrillar material of the cyst wall had become extremely loose in many cysts (Figure 4-29B). The cytoplasm was closely applied to the cyst wall in all cysts. Detachment of the cytoplasm from the cyst wall was not seen.

4.2.3.3 Discussion of Multiple Chemical Treatment

Transmission electron micrograph examinations of *G. lamblia* cysts treated with multiple chemicals showed extensive damage to the cyst surface and cytoplasm. The results supported the hypothesis that enhanced effectiveness of multiple disinfectants was due to a conditioning of the wall of protozoan cysts by the first oxidant (i.e., chlorine, chlorine dioxide, or ozone) so that the secondary oxidant (i.e., chlorine, chlorine dioxide, and monochloramine) can penetrate the cyst more easily (Liyanage *et al.* 1997c). In other words, synergy was the result of increased penetration of a second chemical through a cell membrane whose permeability has been increased by the first chemical (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c).

It was interesting that free chlorine followed by ozone treatment resulted in greater cytoplasmic damage than treatment with the same levels of chemicals in the reverse

order. This observation agrees with the cyst viability measurement studies. Although ozone is a stronger oxidant than free chlorine, it must overcome the barrier of the cyst wall before it can induce damage to the internal organelles. The sugar component of the outer portion of the *Giardia* spp. cyst wall was found to be predominantly galactosamine in the form of N-acetylgalactosamine (Jarroll *et al.* 1989b). This polysaccharide accounts for many cyst wall properties, such as its physical strength and resistance to chlorine and other chemical agents (Aley and Gillin 1995). Perez *et al.* (1995) showed that N-acetyl glucosamine, a simpler sugar present in the peptidoglycan of bacterial cell walls, in viral capsids, as well as in cyst walls of encysted protozoa, was resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacted relatively fast with ozone, but glucose was relatively resistant to degradation. Pre-treatment of cysts with chlorine facilitated penetration of ozone into the cyst cytoplasm. Ozone can react with intracellular components without having to overcome the cyst wall barrier. Sykes (1965) reported that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant. This may explain why ozone as a second disinfectant was more powerful than free chlorine.

4.3 General Discussion

In this thesis, the inactivation efficacy and mechanisms of inactivation of two most commonly used chemical oxidants, ozone and free chlorine against *G. lamblia* cysts, were determined. Sequential inactivation using ozone and free chlorine was also examined using different levels of ozone or free chlorine pre-treatment, and CT products of the second chemical treatment. Mechanisms of chemical inactivation of *G. lamblia* cysts were deduced from the morphological analysis of transmission electron microscope images.

The superior efficacy of ozone has made it one of the leading chemical oxidants for controlling *G. lamblia* in drinking water (Jarroll 1988). At room temperature, 2 log-

unit of inactivation required a CT product of about 0.8 mg·min/L (Finch 1993). Using vital dye staining as measurement of viability and *in vitro* derived *G. lamblia* cysts, a CT product of 2.5 mg·min/L was observed from this study for a 2 log-units of inactivation of *G. lamblia* cysts. Results from this study also confirmed that free chlorine was not very effective for *G. lamblia* inactivation. Two log-units of inactivation at room temperature required a CT product of 500 mg·min/L in this study.

Free chlorine is frequently used as the final chemical treatment in water treatment facilities that use ozone as the primary disinfectant. In this study, a significant synergistic effect was observed in the sequential disinfection. For the treatment investigated (less than 0.5 mg/L ozone for 5 minutes), the gross kill of the sequential inactivation increased linearly with the CT products of the second disinfectant. This study also investigated the effect of free chlorine treatment followed by ozone. Under the same conditions, sequential inactivation in this order achieved greater gross inactivation as well as greater synergistic effects. Although this particular sequence has not been previously studied, greater synergy was observed by Li (2001a) in *Cryptospordium parvum*, when free chlorine was used the second disinfectant following ozone pre-treatment than monochloramine. The author suggested that the extent of sub-lethal injury that could cause synergy depending on the oxidant used in the second treatment. A stronger oxidant in the second treatment requires a relative minor injury for synergy.

Electron microscopic studies of *G. lamblia* cysts of varying inactivation levels were investigated. By varying CT products of ozone and chlorine, 3 levels of inactivation (low, moderate, and high) were achieved by ozone or by free chlorine. It was found that ozone and free chlorine elicited similar series of ultrastructural changes in *G. lamblia* cysts. Initially, ozone or free chlorine affected the filamentous layer of the cyst wall, which presumably made the cyst more permeable. The chemical subsequently penetrated into the cyst and damaged the membranous layers of the cyst

wall. Continued entry of the oxidant into the cyst eventually affected the nucleus and other cytoplasmic components.

Alteration of the cyst wall was clearly the first site of lethal action. The results of single oxidant treatments supported previous studies documenting physical damages of cell envelopes and plasma membranes (Dave 1999; Khadre and Yousef 2001; Langlais and Perrine 1986; Perrine *et al.* 1984; Swanson *et al.* 1977; Wickramanayake 1984). Dave (1999) found that treatment of *Salmonella enteritidis* with aqueous ozone disrupted the cell membranes as seen in TEM. Khadre and Yousef (2001) found that spores of *Bacillus subtilis* treated with aqueous ozone showed heavily disrupted outer spore coats. Microscopic observation of inactivation of trophozoites of *Naegleria* and *Acanthamoeba* showed that they were rapidly destroyed and the cell membrane was ruptured (Perrine *et al.* 1984). Langlais and Perrine (1986) showed that ozone affected the plugs in *Naegleria gruberi* cysts. Depending on the ozonation conditions, these plugs were completely removed or were partially destroyed. Electron microscope examinations of injured *Escherichia coli* cells showed morphological changes in the cell envelope following chlorine treatment (Zaske *et al.* 1980). Permeability changes due to chlorine treatment have been documented by many (Chang 1944; Kulikovsky *et al.* 1975; Venkobachar *et al.* 1977; Williams *et al.* 1991). Venkobachar *et al.* (1977) observed the leakage of proteins, RNA, and DNA following treatment of *Escherichia coli* cells with very low doses of chlorine. DNA was found to be released at very high concentrations of chlorine. The authors concluded that chlorine must have damaged the integrity of the cell membrane resulting in a leakage of macro and micro-molecules. Haas and Engelbrecht (1980) supported this in a study using *E. coli*. The authors showed that chlorine promoted cell leakage of potassium ions, total organic carbon, UV-absorbing material, in decreasing order of quantity. It was therefore concluded that lower molecular weight substances are preferentially released. Similar results were seen in more resistant spore forming bacteria (Kulikovsky *et al.* 1975; Williams and Russell 1991). Chlorine treated *Bacillus cereus* spores released nucleic acids, which was

believed to be a consequence of the destruction of the permeability barrier of the organism (Kulikovsky *et al.* 1975). Using the same organism, researchers observed efflux of potassium from the cells and a dramatic increase in hydrogen ion permeability following free chlorine treatment (Williams and Russell 1991).

Electron micrograph examinations in this study also showed that low and moderate doses of ozone or free chlorine resulted in primarily structural damage leading to leakage of small molecules, whereas higher doses resulted in cytoplasmic granulation and dissolution, possibly from oxidation of proteins and nuclei acids of intracellular components. It can be concluded that structural damage to the cyst wall components, a primary lethal event, was reversible; whereas damage to nuclear material and other cytoplasmic metabolic activities, though secondary, was irreversible. This is in agreement with previous morphological studies of necrosis (Arstila and Trump 1969; Dive *et al.* 1992; Ginn *et al.* 1969).

Morphological changes following chemical treatment were comparable with those of excystation (Buchel *et al.* 1991a; Buchel *et al.* 1987; Coggins and Schaefer 1984; Coggins and Schaefer 1986). In this study, it was observed that ozone and free chlorine elicited similar cellular responses (at the structural level) to those observed after induction of excystation. One possible mechanism of inactivation by oxidants could be a pathway similar to excystation. During excystation, the protective shell of the parasite disintegrates. Iin this state, the excysted organism has little defense against oxidation or potential exposure to osmotic pressure changes.

Sequential experiments in this study gave morphological support to the hypothesis that the first chemical disinfectant pre-conditions the parasite by making the cyst wall more permeable for subsequent entry of the second disinfectant (Finch and Li 1999; Finch *et al.* 2000b; Gyürék *et al.* 1996; Li *et al.* 1999; Li *et al.* 2001b; Liyanage *et al.* 1997a; Liyanage *et al.* 1997b; Liyanage *et al.* 1997c). Extensive cytoplasmic damage was observed in addition to cyst surface alterations, even with minimal levels of the

second disinfectant treatment. Furthermore, morphological studies paralleled results of the inactivation studies. *G. lamblia* cysts treated with free chlorine followed by ozone showed greater damages in the cytoplasm than cysts treated with similar levels of the same oxidants in reverse order. Sykes (1965) reported that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant. It is not surprising the subsequent entry of cyst wall by ozone created more damage than free chlorine.

Characterization of the nature of oxidant action has significance in the optimization of water treatment designs and protocols. This study showed that the key step in chemical inactivation of encysted protozoan parasites was overcoming the barriers of the cyst walls. Oxidant doses and contact times must be applied to induce sufficient damage in the cyst walls in order to be effective. Furthermore, the results from this study provided ultrastructural basis for the difference in efficacies of the chemical oxidants: ozone and free chlorine. Finally, the sequence of chemical addition has been shown to make a great difference in the final inactivation levels of microorganisms. When ozone and free chlorine are used in water treatment, it is more effective to apply free chlorine followed by ozone.

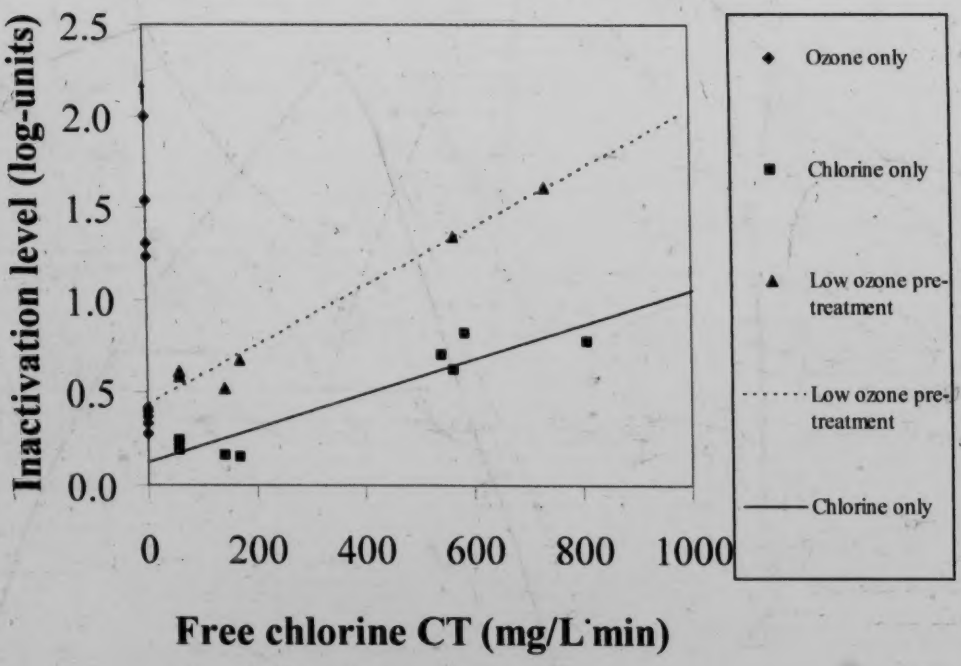

Figure 4-1. Inactivation of *G. lamblia* cysts as a function of chlorine CT in oxidant demand-free 0.05 M phosphate buffer at pH 6 and 5 °C

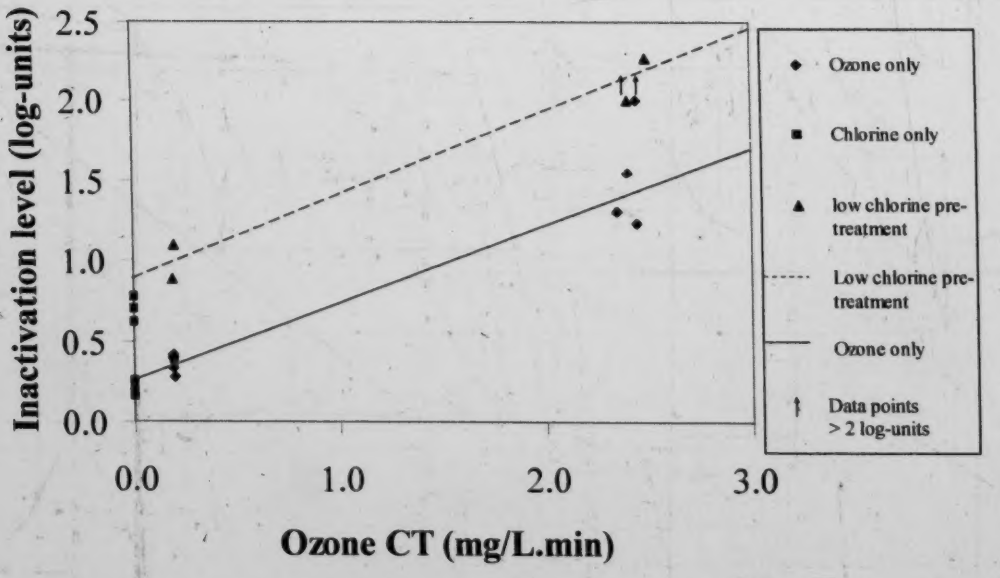

Figure 4-2. Inactivation of *G. lamblia* cysts as a function of ozone CT in oxidant demand-free 0.05 M phosphate buffer at pH 6 and 5 °C

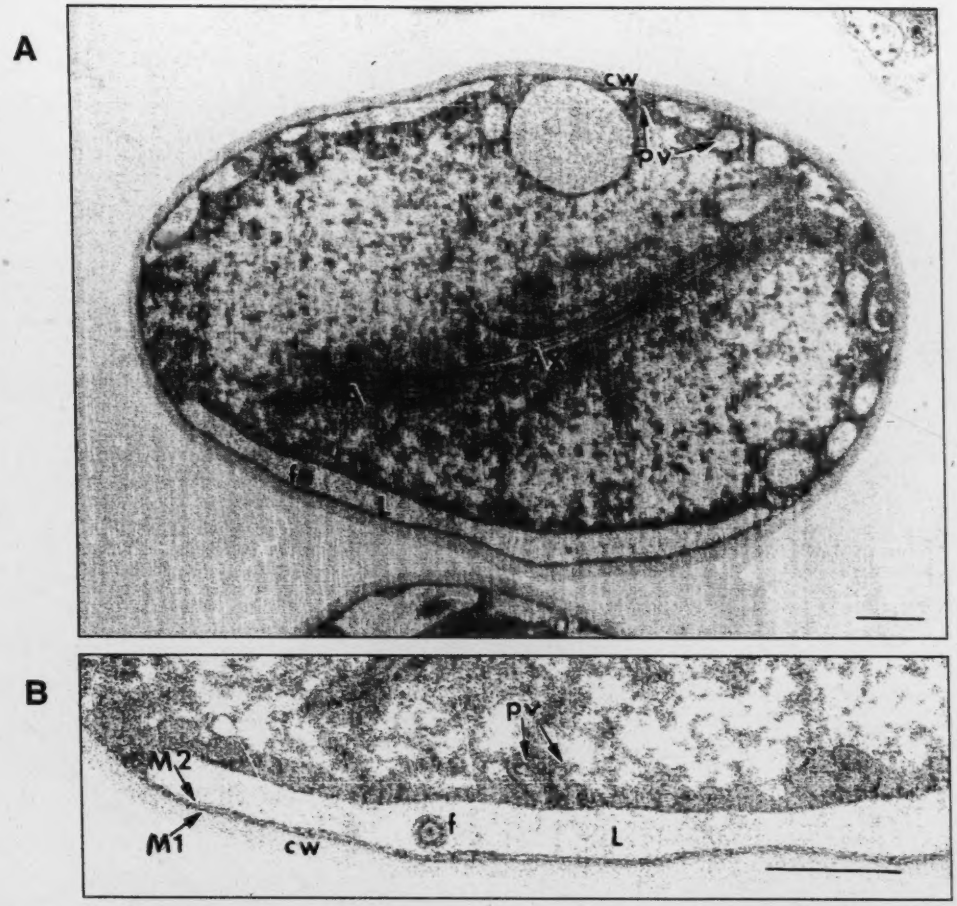

Figure 4-3. Transmission electron micrographs of untreated *G. lamblia* cyst. A. Longitudinal section showing cyst wall (cw), axonemes of flagella (A), axostyle (Ax), free flagella (f) in the lacunar space (L), and peripheral vacuoles (pv). X 8,900 Bar = 1μm B. High magnification of cyst wall, showing fibrillar components (cw), the membrane attached to the fibrillar component (M1), and the membrane surrounding the trophozoite (M2). X 19,000 Bar = 1μm

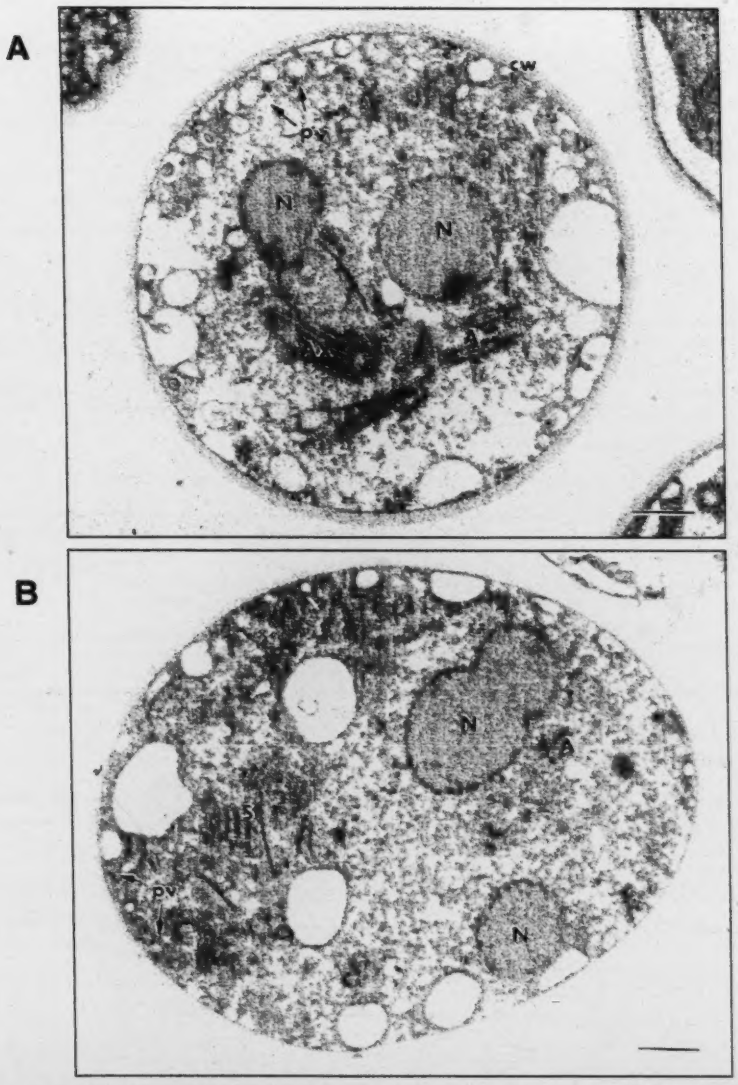

Figure 4-4. Transmission electron micrographs of untreated *G. lamblia* cysts. A. Cross section showing cyst wall (cw), peripheral vacuoles (pv), nuclei (N); Oblique section through the axostyle (AX) shows fan-shaped distribution of microtubules in association with axonemes (A). X 19,000 Bar = 1μm B. Cross section showing nuclei (N), microtubule-ribbon complexes of striated disk fragments (S) scattered in the cytoplasm, peripheral vacuoles (pv), and axonemes (A). X 12,000 Bar = 1μm

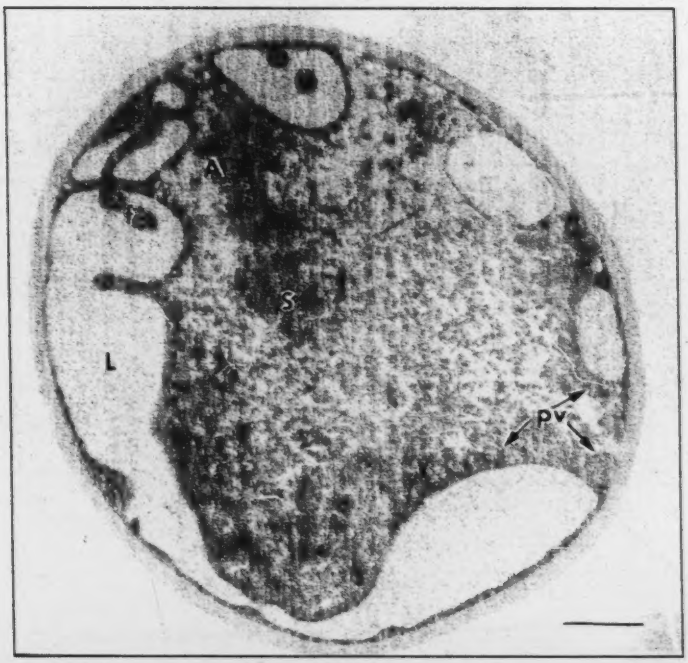

Figure 4-5. Transmission electron micrograph of untreated *G. lamblia* cysts. Cross section showing nuclei (N), microtubule-ribbon complexes of striated disk fragments (S) scattered in the cytoplasm, peripheral vacuoles (pv), and axonemes (A). X 12,000 Bar = 1μm

Figure 4-6. Transmission electron micrograph of *G. lamblia* cyst treated with low ozone doses. Note that fibers of the cyst wall (cw) are loosely bound; membranes of the cyst wall are ruffled (arrow). A. Cysts treated with 0.1 mg/L ozone for 30 sec at pH 6 and 25 °C. X 10,000 Bar = 1μm B. Cysts treated with 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. X 12,000 Bar = 1μm

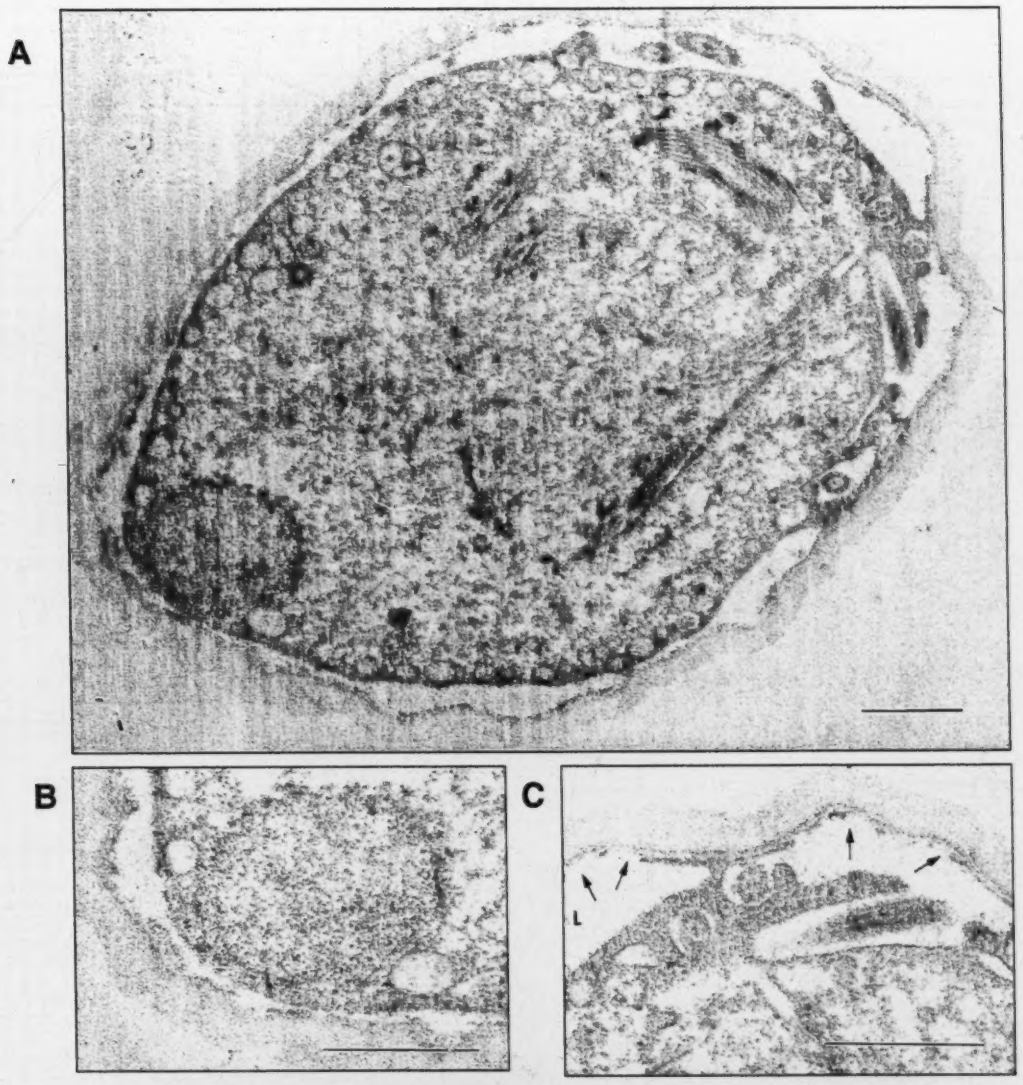

Figure 4-7. *G. lamblia* cysts treated with 0.5 mg/L ozone for 2 min at pH 6 and 25 °C. A. Thin section showing detachment of parasite from the cyst wall. X 14,000 Bar = 1μm B. High magnification of nucleus showing increased electron density around the nuclear periphery. X 30,000 Bar = 1μm C. High magnification of the cyst wall showing loosening of cell wall fibers, damaged membranes underlying the cyst wall (arrow), and enlarged lacunar space (L). X 30,000 Bar = 1μm

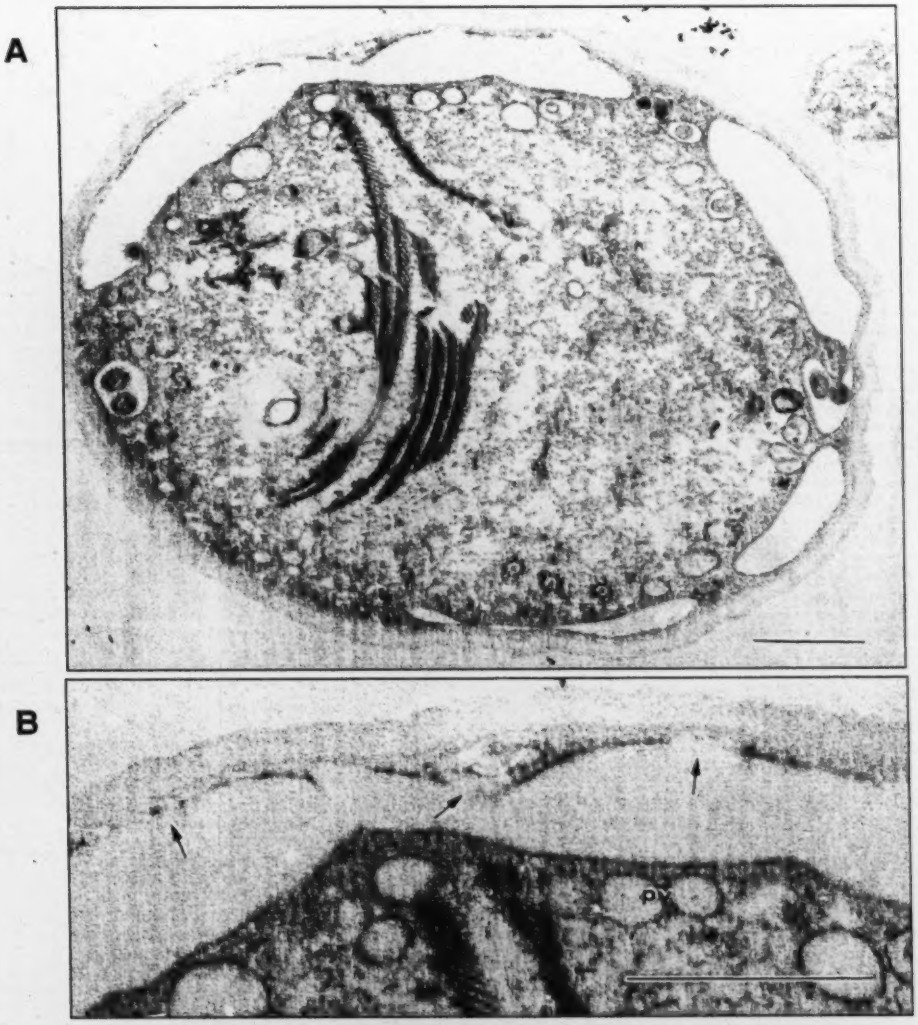

Figure 4-8. A. Transmission electron micrograph of *G. lamblia* cysts treated with 0.5 mg/L ozone for 2 min at pH 6 and 25 °C. X 18,000 Bar = 1μm B. High magnification of the cyst wall showing damaged membranes underlying the cyst wall (arrow), and intact peripheral vacuoles (pv). X 38,000 Bar = 1μm

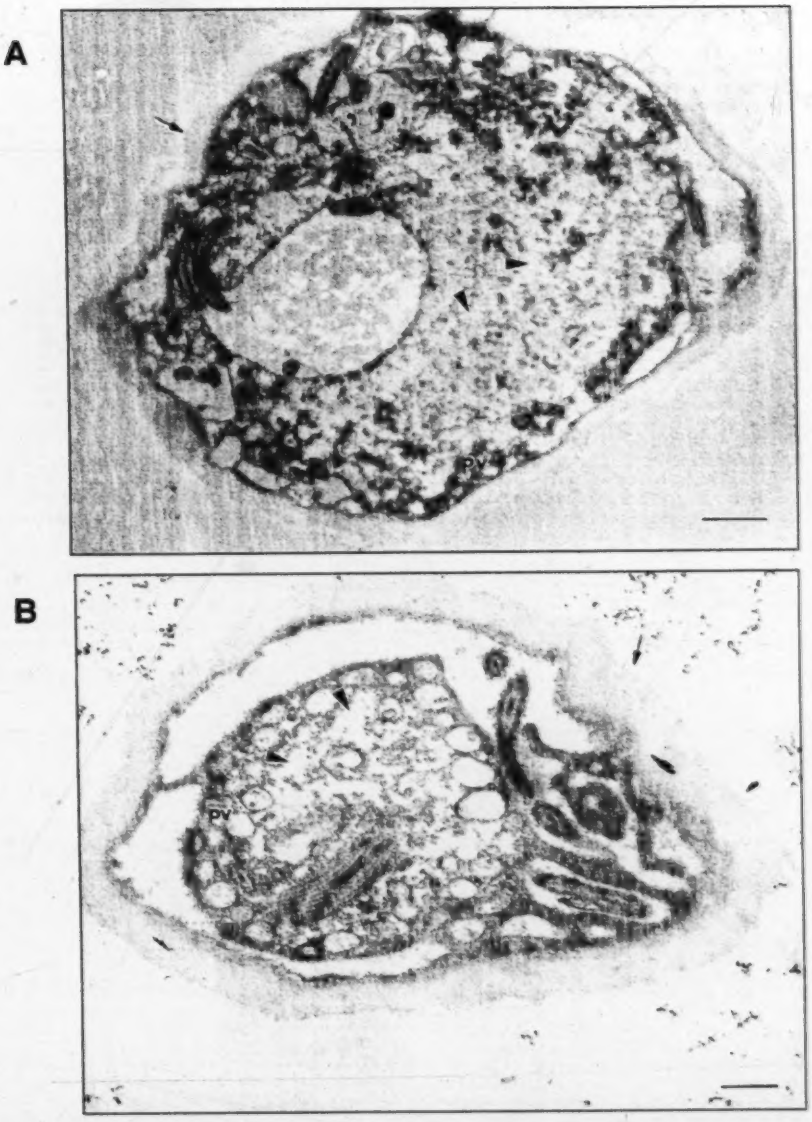

Figure 4-9. Transmission electron micrographs of *G. lamblia* cysts treated with 0.5 mg/L ozone for 2 min at pH 6 and 25 °C, showing thickening of the cyst wall (long arrow), damage to peripheral vacuoles (pv), and disintegration of cytoplasmic material (short arrow). A. X 12,000 Bar = 1μm B. X 11,000 Bar = 1μm

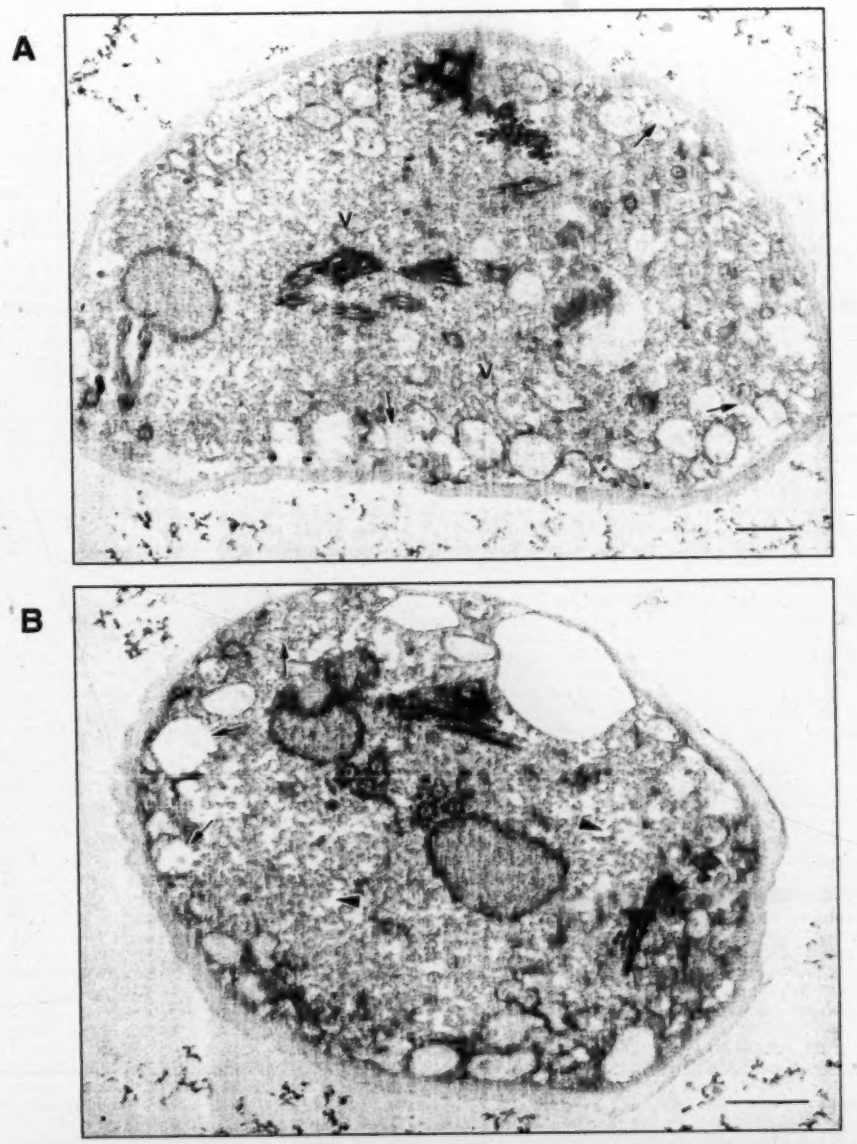

Figure 4-10. Transmission electron micrographs of *G. lamblia* cysts treated with 0.5 mg/L ozone for 5 min at pH 6 and 25 °C. Note damage to peripheral vacuoles (small arrow), formation of internal vacuoles (V), and development of honeycombed appearance of the cytoplasm (large arrow). A. X 11,000 Bar = 1μm B. X 14,000 Bar = 1μm

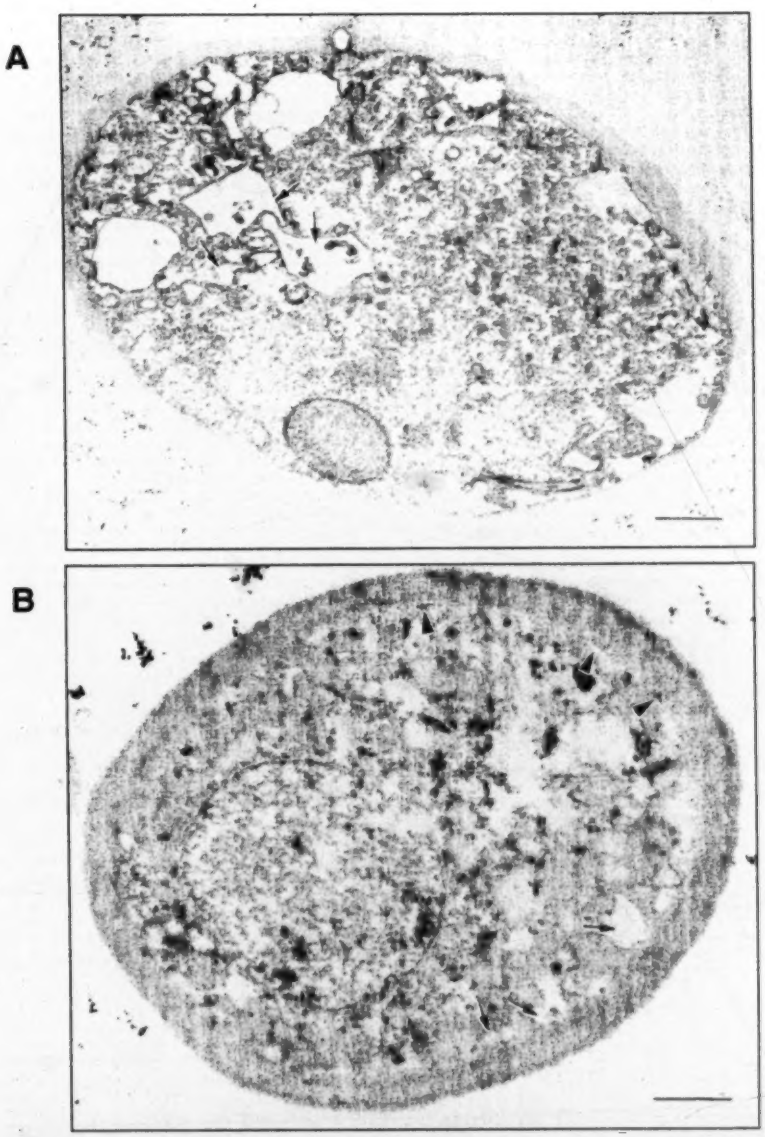

Figure 4-11. Transmission electron micrographs of *G. lamblia* cyst treated with 0.5 mg/L ozone for 5 min at pH 6 and 25 °C. A. Note the disorganized and irregularly shaped internal vacuoles (long arrow). X 12,000 Bar = 1μm B. Note remnants of the membranes along the inner cyst wall (short arrow), circular to oval structures with low electron densities at the periphery resembling peripheral vacuoles (long arrow), and broken nuclear membrane. X 14,000 Bar = 1μm

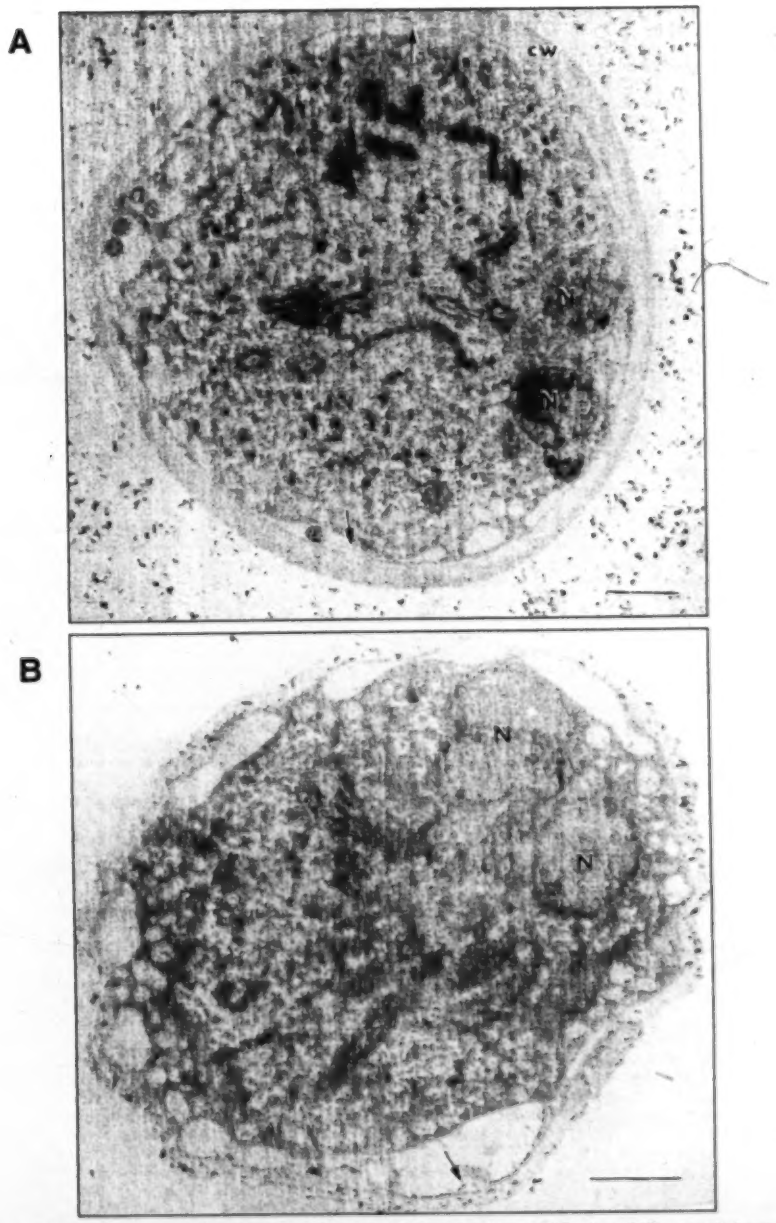

Figure 4-12. Transmission electron micrographs of *G. lamblia* cyst treated with 2.0 mg/L free chlorine for 30 min at pH 6 and 25 °C. A. Note thickened cyst wall (cw), damaged cyst wall membranes (arrow), and increase in density of nuclear material (N). X 12,000 Bar = 1μm B. Note blebbing on cyst wall, broken cyst wall membranes (arrow), and intact nuclei (N). X 16,000 Bar = 1μm

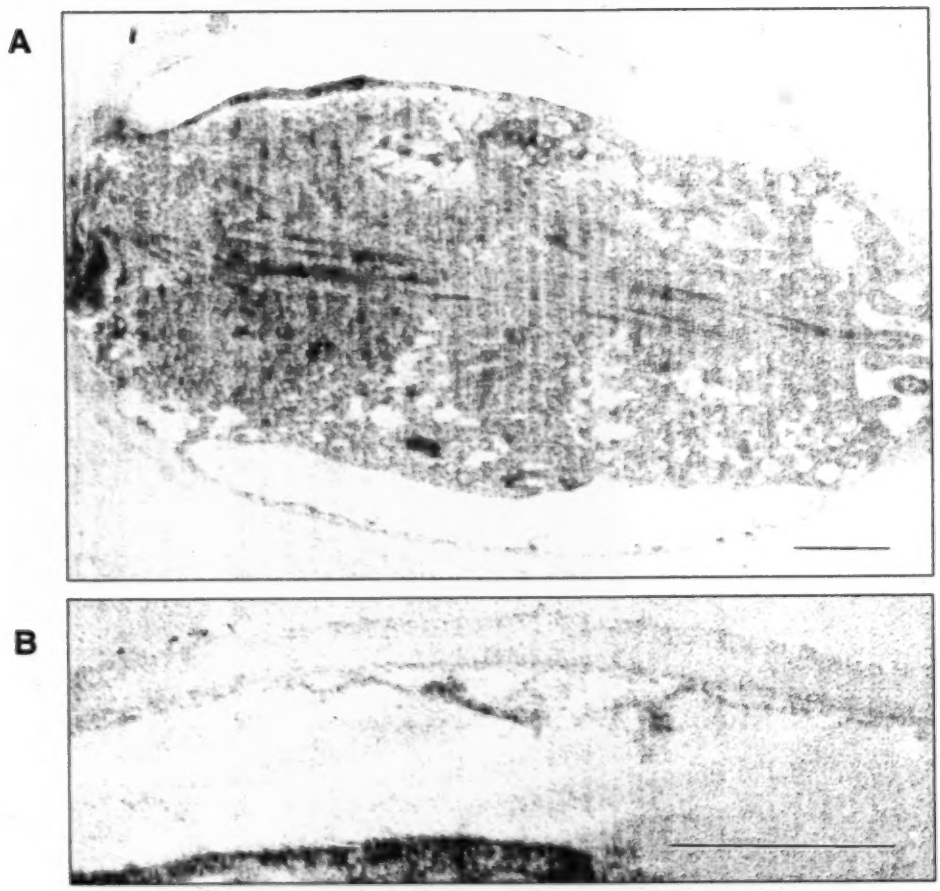

Figure 4-13. Transmission electron micrographs of *G. lamblia* cysts treated with 2.0 mg/L free chlorine for 30 min at pH 6 and 25 °C. A. Note the detachment of the parasite from the cyst wall layers. X 15,000 Bar = 1μm B. High magnification of cyst wall, showing loosening of cyst wall fibers, damaged cyst wall membranes, and enlargement of lacunar space. X 34,000 Bar = 1μm

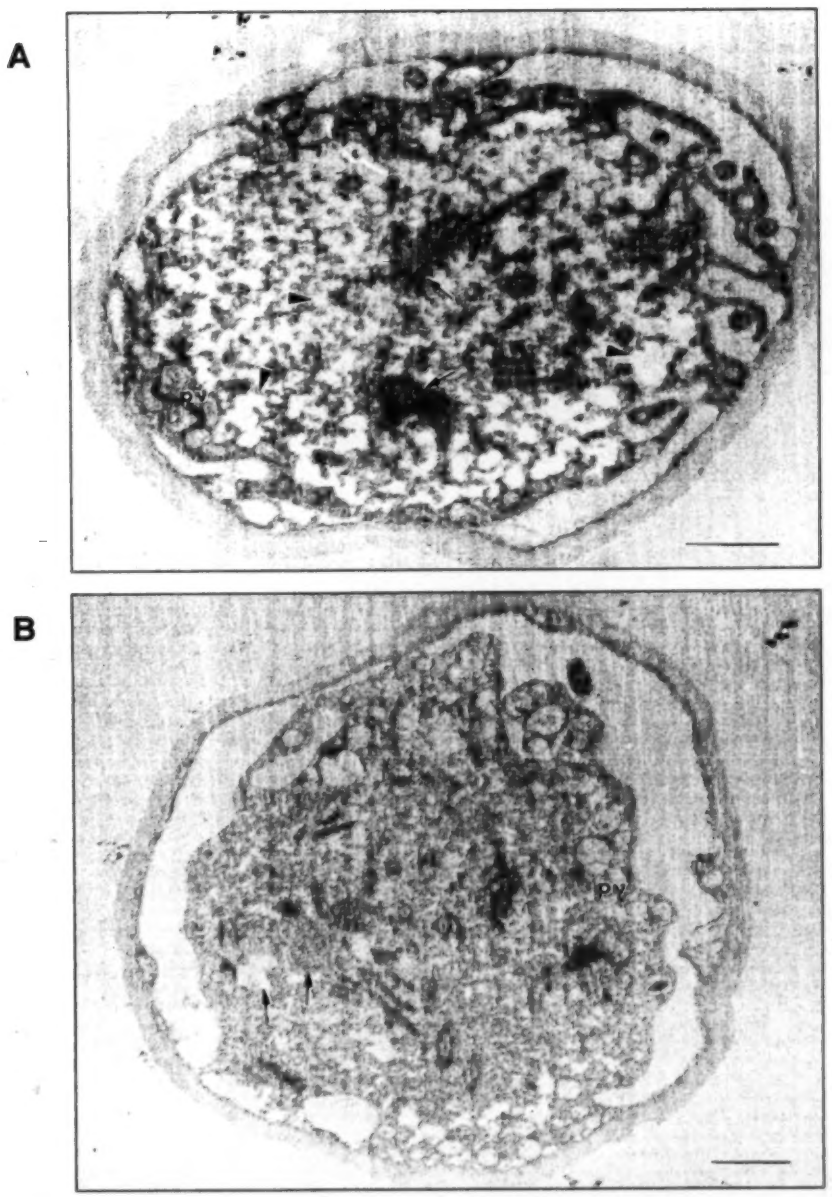

Figure 4-14. Transmission electron micrographs of *G. lamblia* cyst treated with 2 mg/L free chlorine for 100 min at 25 °C and pH 6. Note intact peripheral vacuoles, damages to the nucleus (long arrow), and development of honeycombed appearance of the cytoplasm (short arrow). A. X 17,000 Bar = 1μm B. X 13,000 Bar = 1μm

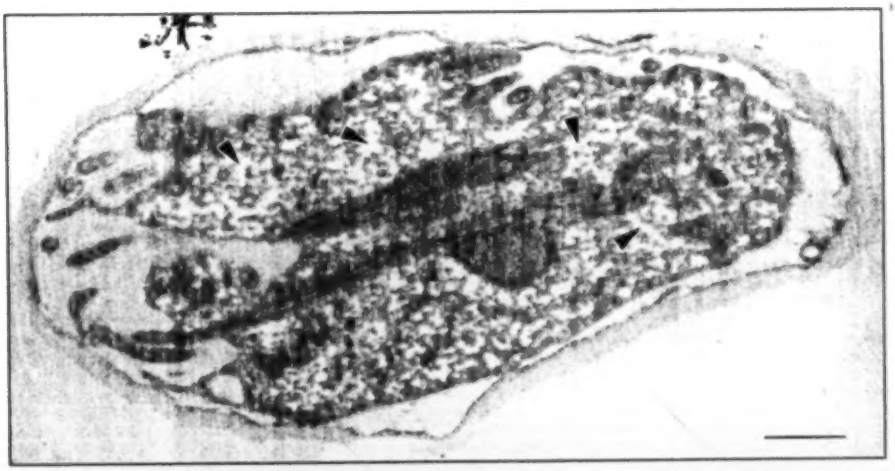

Figure 4-15. Transmission electron micrograph of *G. lamblia* cyst treated with 2 mg/L free chlorine for 100 min at 25 °C and pH 6. Note the change in cyst shape, enlargement of the lacunar space, honeycombed appearance (arrow) of the cytoplasm. X 12,000 Bar = 1μm

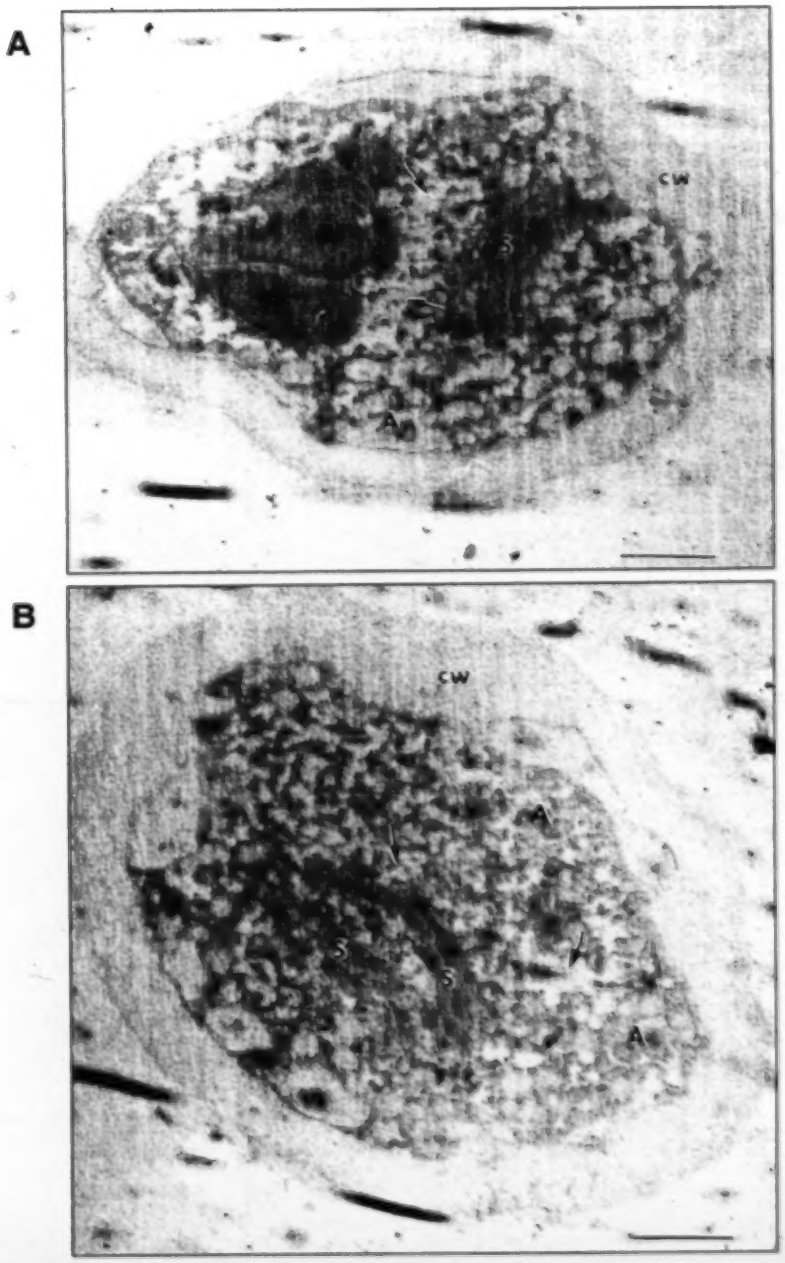

Figure 4-16. Transmission electron micrographs of *G. lamblia* cysts treated with 5 mg/L chlorine for 120 min at 25 °C and pH 6, showing swollen cyst wall (cw), disintegrated cytoplasm (arrows), loss in sharp details of striated disk fragments (S), and axonemes (A). A. X 15,000 Bar = 1μm B. X 15,000 Bar = 1μm

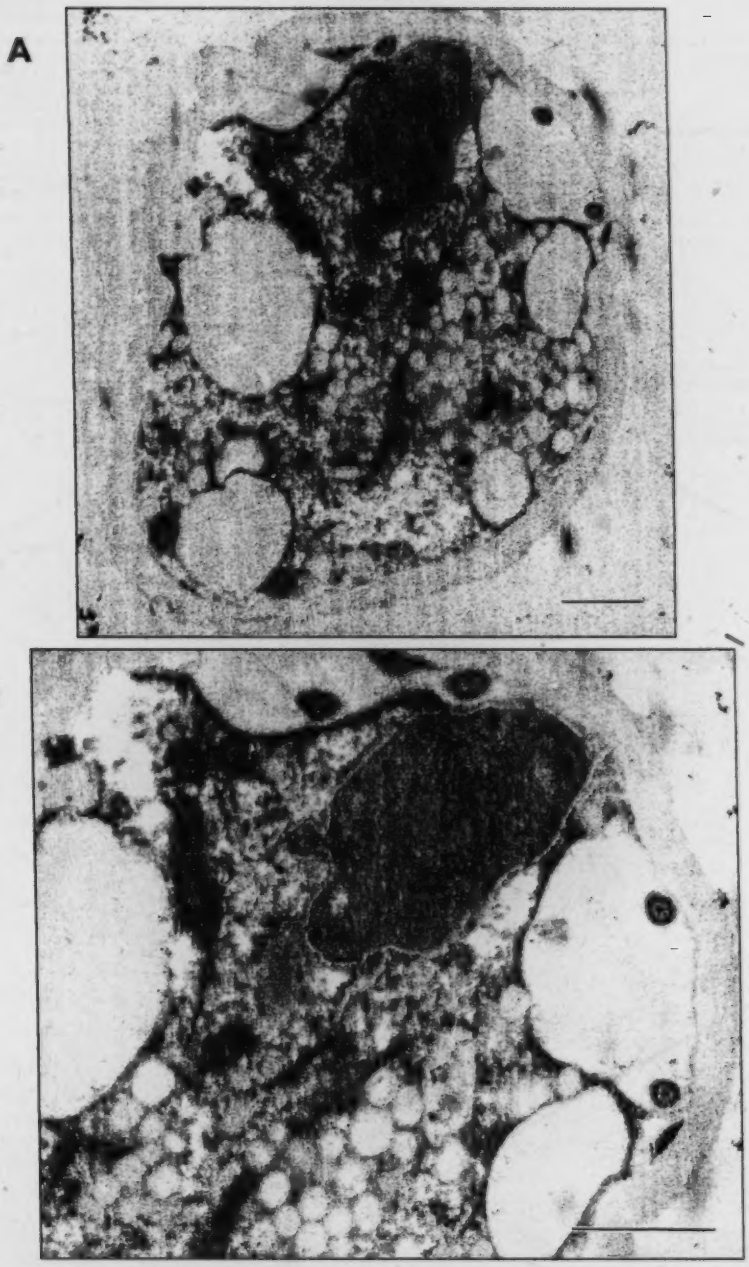

Figure 4-17. *G. lamblia* cyst treated with 0.2 mg/L ozone for 1 min followed by 2mg/L free chlorine for 30 min at pH 6 and 5 °C. X 12,000 Bar = 1μm B. High magnification of nucleus showing alteration in shape. X 23,000 Bar = 1μm

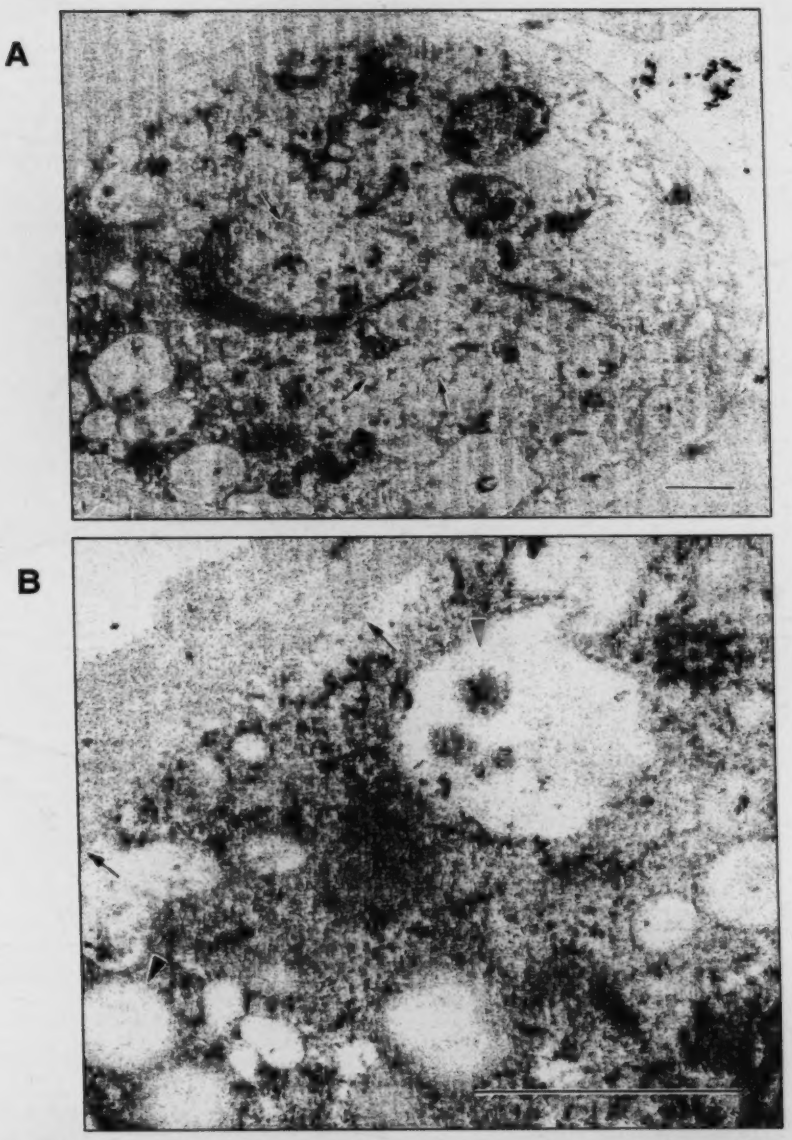

Figure 4-18. ***G. lamblia*** **cyst treated with 0.2 mg/L ozone for 1 min followed by 2mg/L free chlorine for 30 min at pH 6 and 5 °C. A. Note condensation of nuclear material, damage of internal vacuoles (arrow). X 12,000 Bar = 1μm B. High magnification of cyst wall showing loosening of cyst wall fibers, remnants of membranes underlying the cyst wall (small arrow), peripheral vacuoles (PV) lacking their characteristic membrane (large arrow). X 37,000 Bar = 1μm**

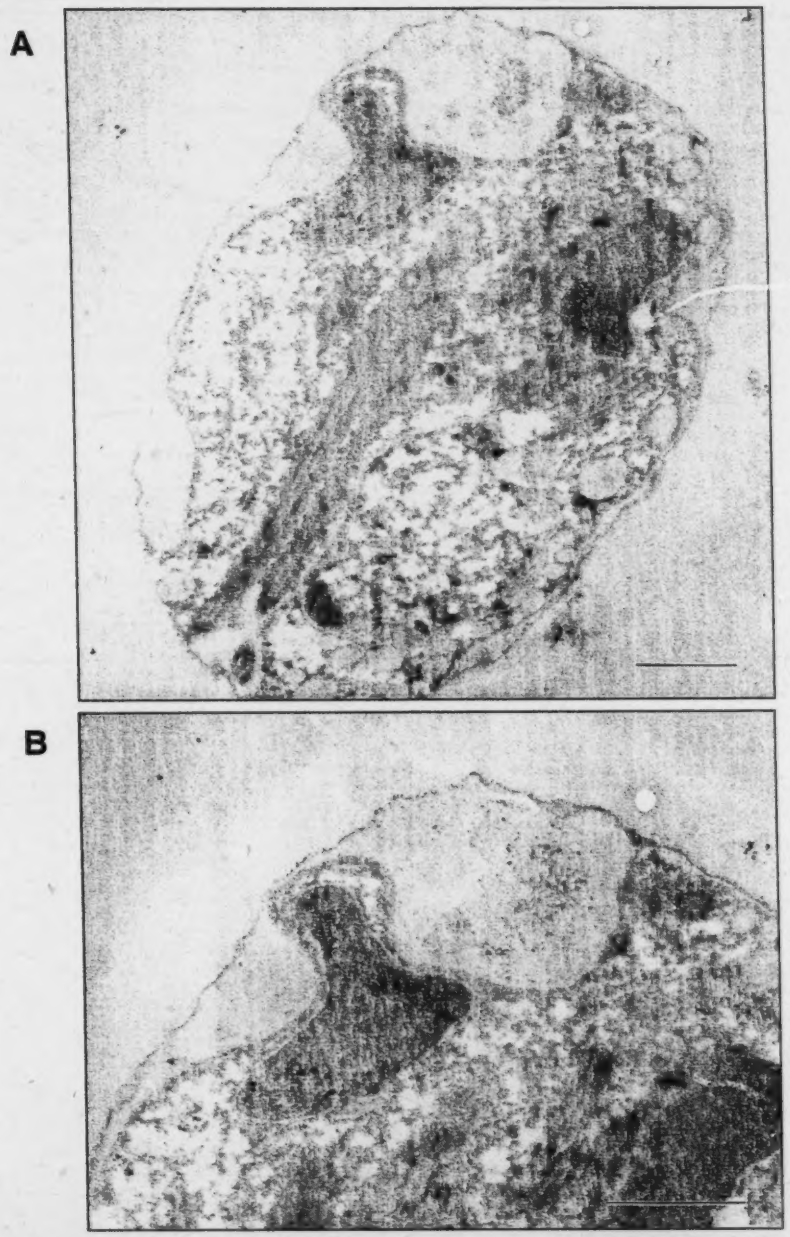

Figure 4-19. *G. lamblia* cyst treated with 0.2 mg/L ozone for 1 min followed by 2 mg/L free chlorine for 30 min at pH 6 and 5 °C. Note that microfibrils of the cyst wall have lost staining properties. A. X 15,000 Bar = 1μm B. X 27,000 Bar = 1μm

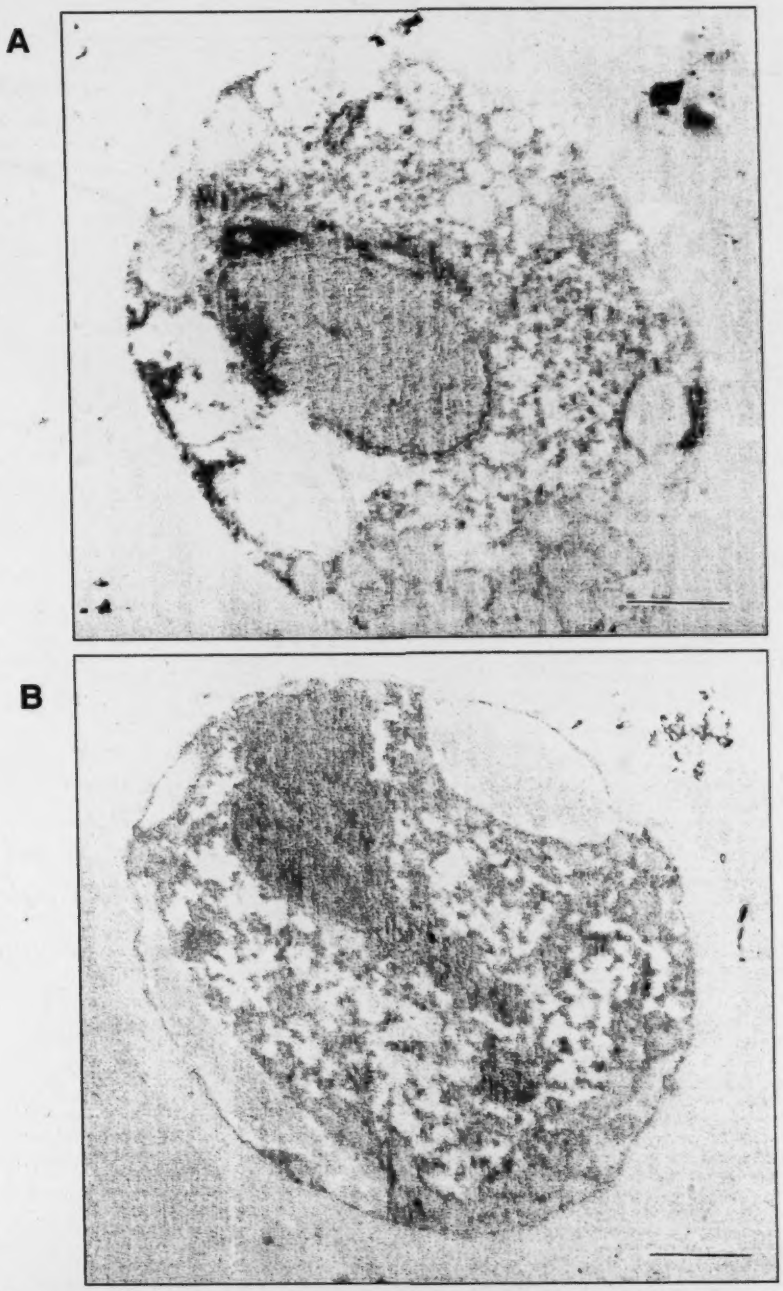

Figure 4-20. Transmission electron micrographs of *G. lamblia* cyst treated with 0.2 mg/L ozone for 1 min followed by 5 mg/L free chlorine for 120 min at pH 6 and 5 °C. A. X 15,000 Bar = 1μm B. X 15,000 Bar = 1μm

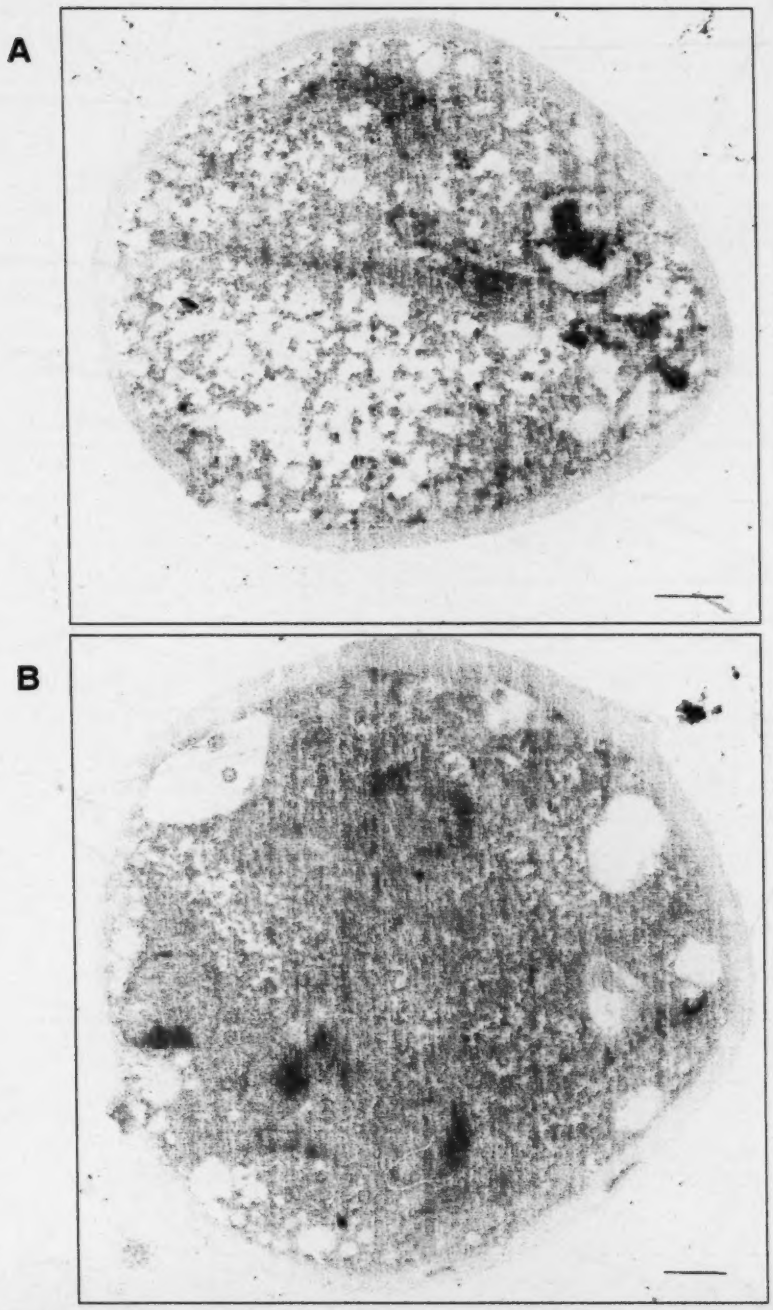

Figure 4-21. Transmission electron micrographs of *G. lamblia* cyst treated with 0.5 mg/L ozone for 5 min followed by 2 mg/L free chlorine for 30 min at pH 6 and 5 °C. A. X 9,000 Bar = 1μm B. X 9,000 Bar = 1μm

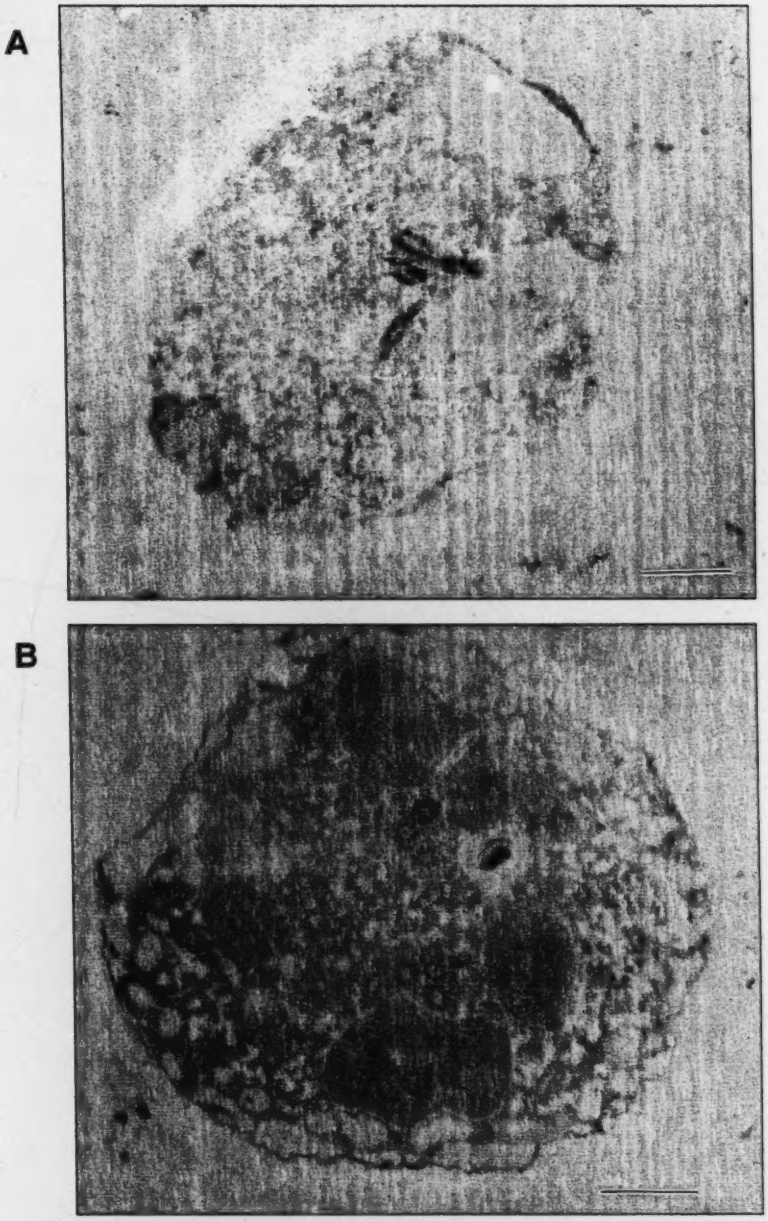

Figure 4-22. Transmission electron micrographs of *G. lamblia* cyst treated with 0.5 mg/L ozone for 5 min followed by 5 mg/L free chlorine for 120 min at pH 6 and 5 °C. A. X 14,000 Bar = 1μm B. X 18,000 Bar = 1μm

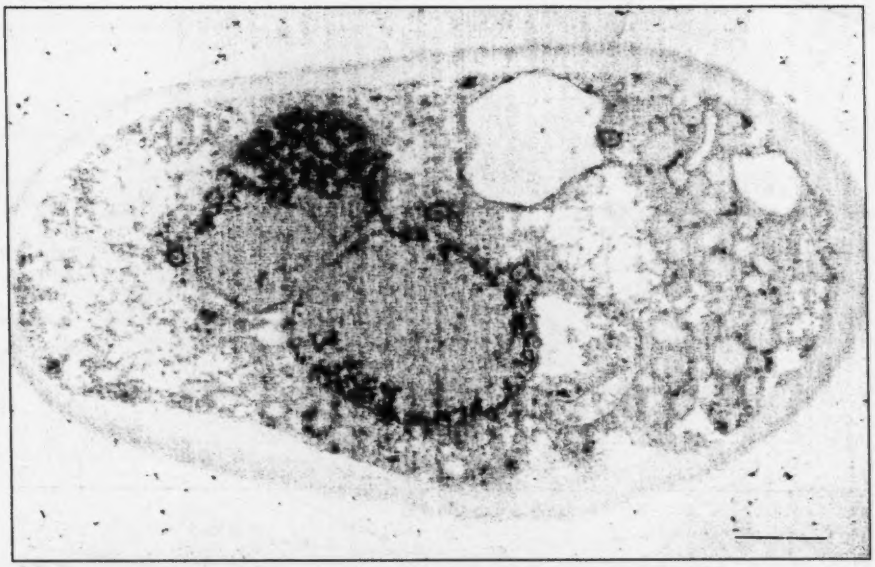

Figure 4-23. Transmission electron micrograph of *G. lamblia* cyst treated with 2 mg/L free chlorine for 30 min followed by 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. X 14,000 Bar = 1μm

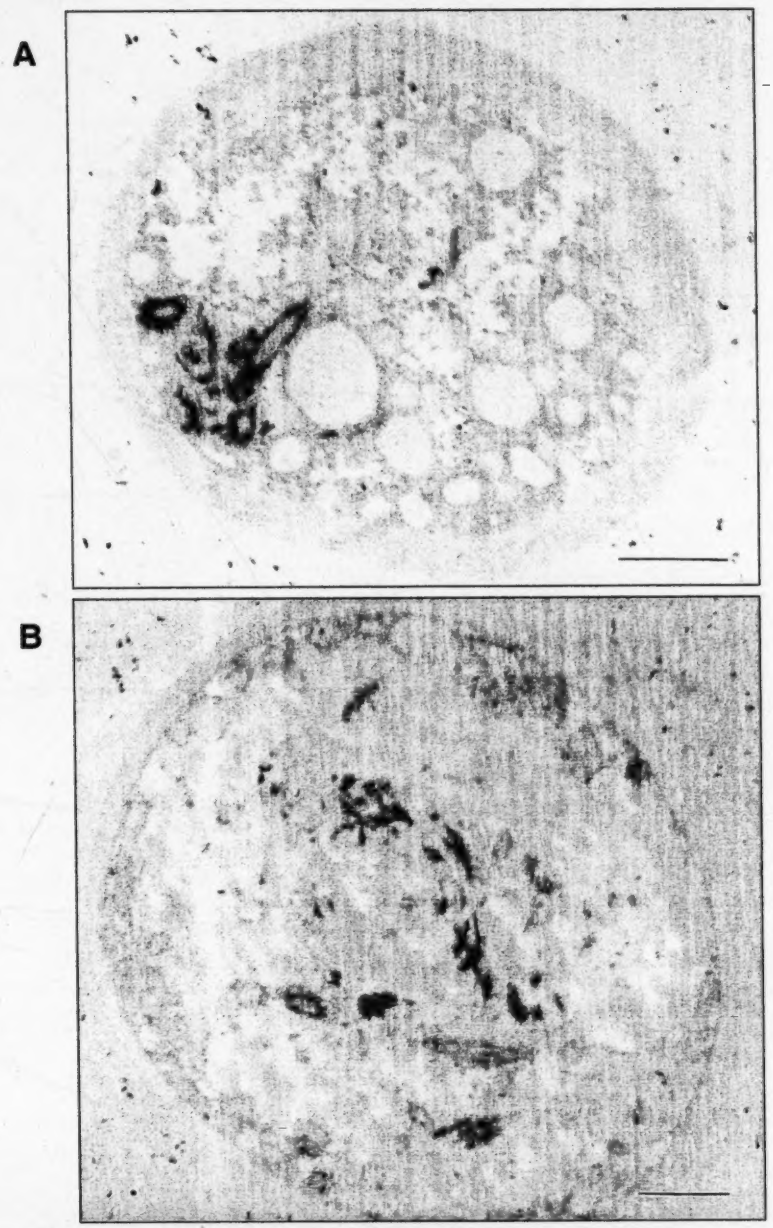

Figure 4-24. Transmission electron micrograph of *G. lamblia* cyst treated with 2 mg/L free chlorine for 30 min followed by 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. A. X 18,000 Bar = 1μm B. X 15,000 Bar = 1μm

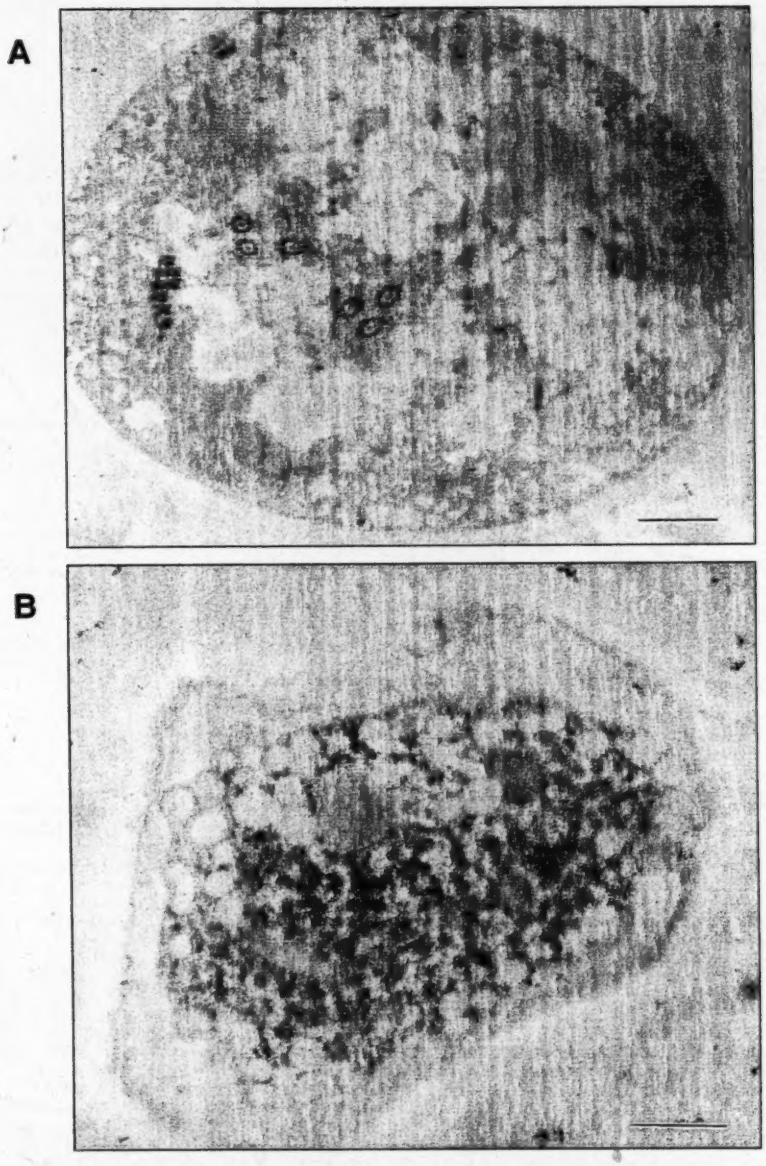

Figure 4-25. Transmission electron micrograph of *G. lamblia* cyst treated with 2 mg/L free chlorine for 30 min followed by 0.5 mg/L ozone for 5 min at pH 6 and 5 °C. A. X 14,000 Bar = 1μm B. X 17,000 Bar = 1μm

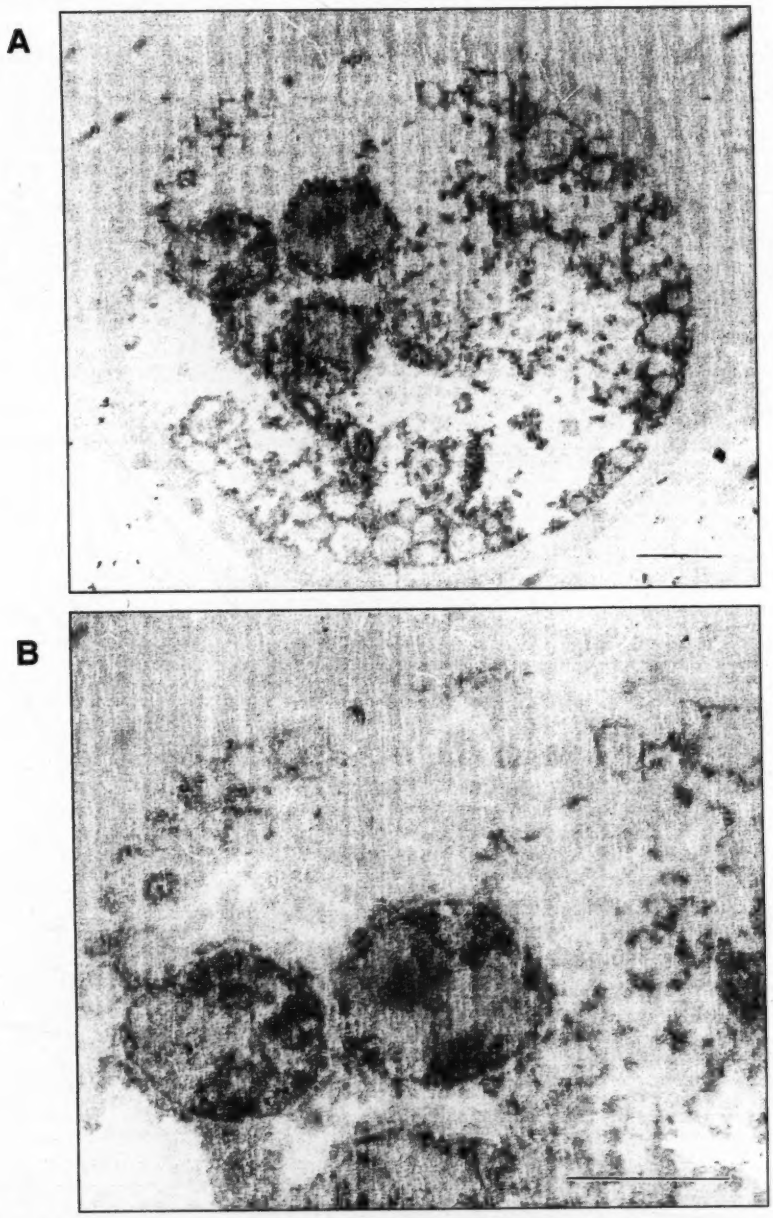

Figure 4-26. Transmission electron micrographs of *G. lamblia* cyst treated with 5 mg/L free chlorine for 120 min followed by 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. A. X 14,000 Bar = 1μm B. X 27,000 Bar = 1μm

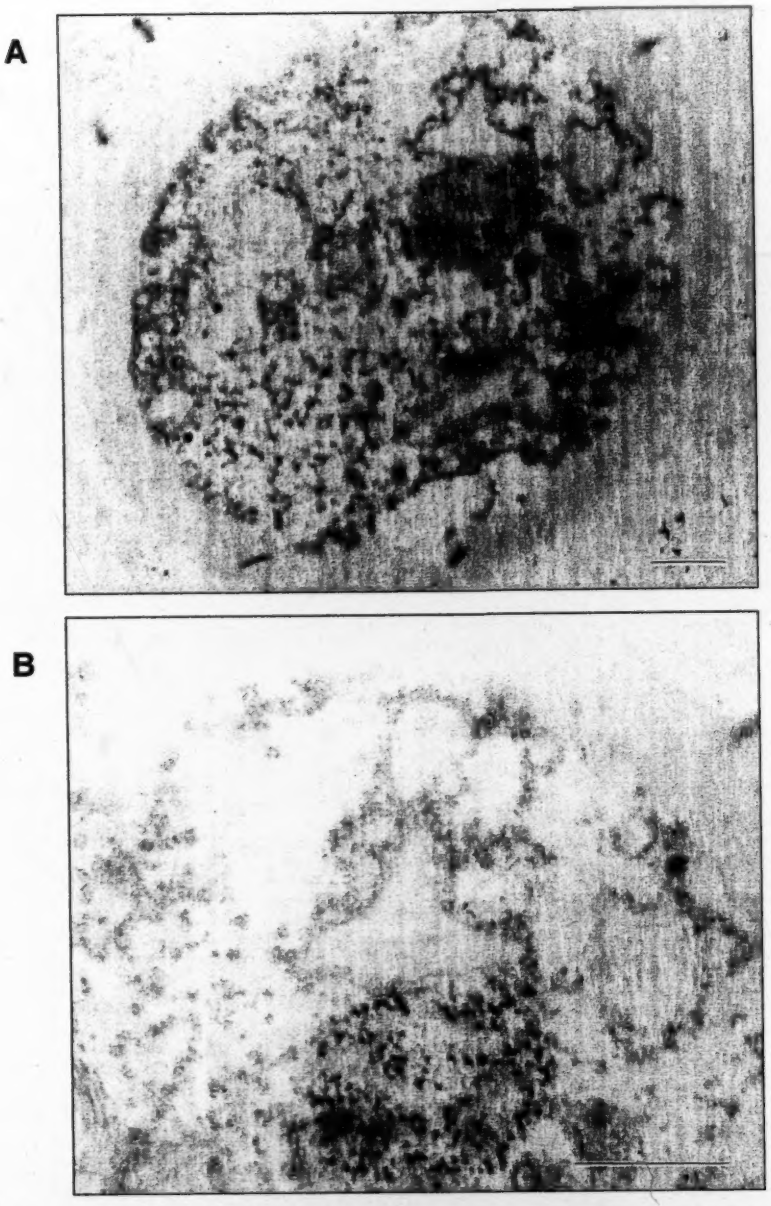

Figure 4-27. Transmission electron micrographs of *G. lamblia* cyst treated with 5 mg/L free chlorine for 120 min followed by 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. A. X 13,000 Bar = 1μm B. X 27,000 Bar = 1μm

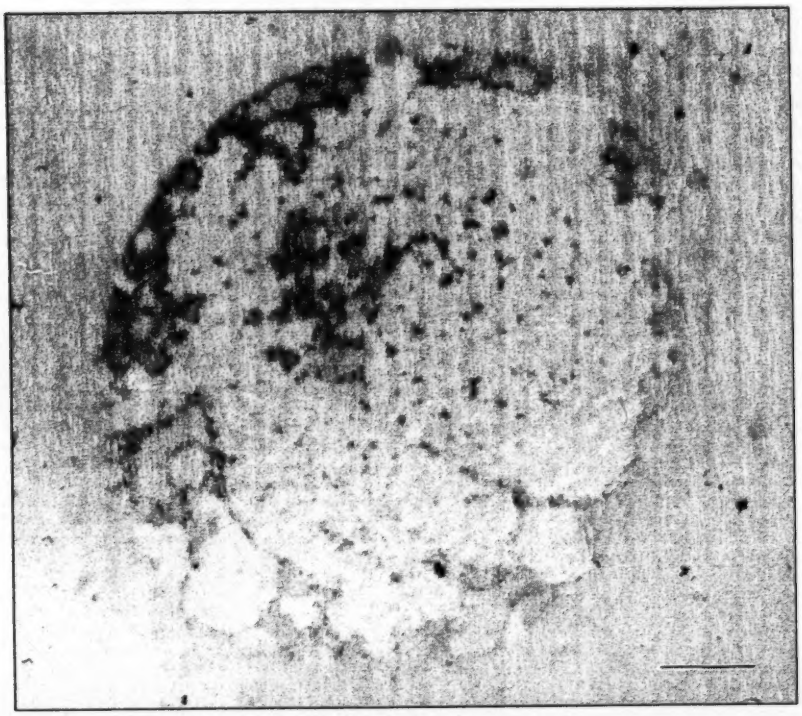

Figure 4-28. Transmission electron micrograph of *G. lamblia* cyst treated with 5 mg/L free chlorine for 120 min followed by 0.2 mg/L ozone for 1 min at pH 6 and 5 °C. X 14,000 Bar = 1μm

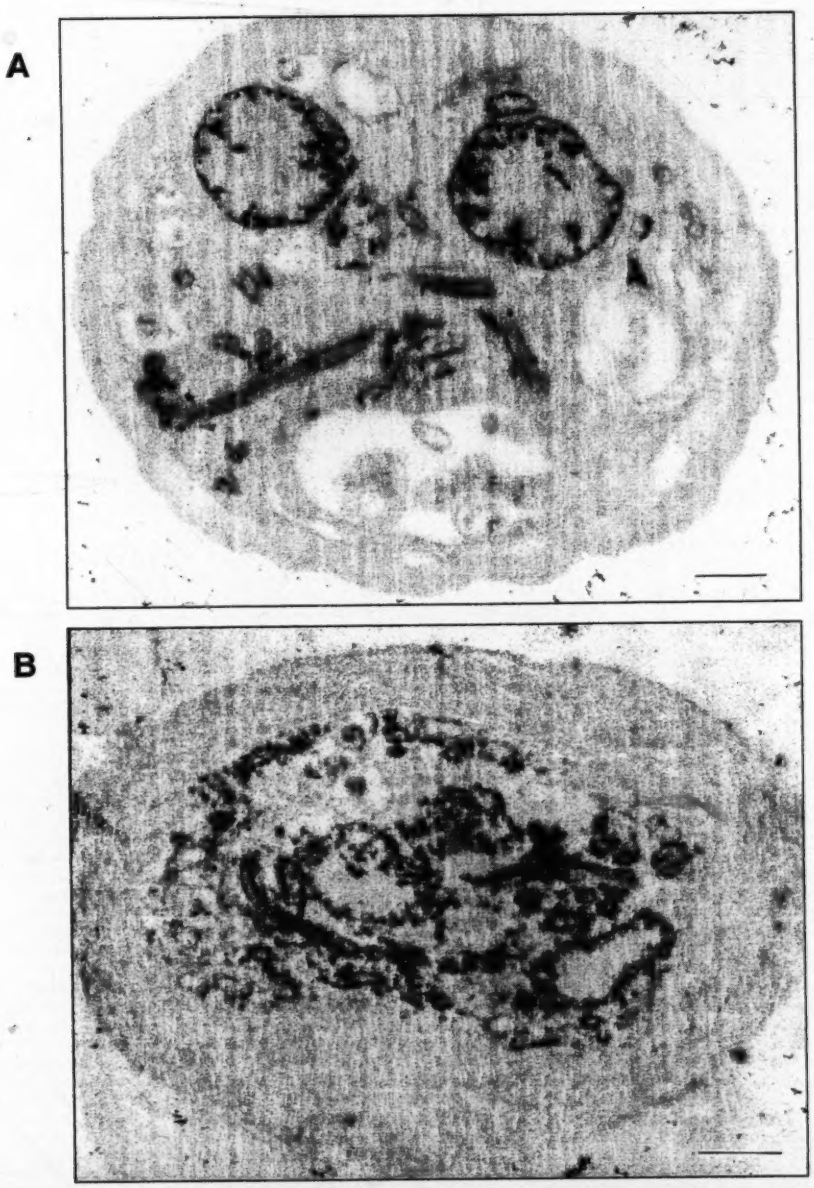

Figure 4-29. Transmission electron micrographs of *G. lamblia* cyst treated with 5 mg/L free chlorine for 120 min followed by 0.5 mg/L ozone for 5 min at pH 6 and 5 °C. A. Note nuclei of a cyst still seen intact even at this level of inactivation. X 12,000 Bar = 1μm B. Note gross loosening of cyst wall fibers. X 14,000 Bar = 1μm

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

From the study of morphology of *G. lamblia* cyst inactivation following single or combined disinfectant chemicals, the following conclusions have been reached:

1. There are similarities in the mechanisms of inactivation by ozone and by free chlorine.
    a. The first site of lethal action is the cyst wall of *G. lamblia*. Both ozone and free chlorine changes the permeability of the cyst wall by loosening of cyst wall microfibrils and breaking membranes underlying the cyst wall and surrounding the parasite.
    b. Alterations to the cyst wall result in leakage of cytoplasmic material as evidenced by the enlargement of the lacunar space, and facilitate the penetration of ozone or free chlorine into the cytoplasm causing internal damages.
2. There are also unique differences between ozone and free chlorine mechanisms of inactivation.
    a. Nuclear damage is an early event in chlorinated cysts, but a late lethal event in ozone treated cysts.
    b. Free chlorine seems to result in aggregation of cytoplasmic materials whereas ozone results in dissolution.
    c. Ozonation results in extensive internal vacuolization, a phenomenon not observed in chlorinated cysts. One hypothesis could be that ozone damages endomembrane systems including the endoplasmic reticulum, an event characterized as "the point of no return" in the process of cell death by Ginn (1969). If true, then the commitment to cell death is more prominent in ozone treated cysts than free chlorine treated cysts.
3. There are comparable morphological developments in chemically treated cysts and cysts induced by excystation stimuli. Both processes result in the

loosening of cyst wall, lysis of peripheral vacuoles, and the enlargement of the lacunar space. It is possible that *G. lamblia* cysts are inactivated by chemicals via an excystation pathway.

4. Synergy was observed for ozone followed by free chlorine treatment of *G. lamblia* cysts at pH 6 and 5 °C. The level of synergy was related to the level of ozone pre-treatment and CT products of free chlorine. The gross kill and synergistic effect increased linearly with the CT products of the free chlorine treatment.
5. Greater synergy was observed for free chlorine followed by ozone treatment of *G. lamblia* cysts at pH 6 and 5 °C. The level of synergy was related to the level of ozone post-treatment and CT products of free chlorine. The gross kill and synergistic effects increased linearly with the CT products of the free chlorine treatment. It is concluded that the use of a stronger second oxidant results in greater synergy.

5.2 Recommendations

Despite the findings reported in this study, several issues remain to be addressed. It is recommended that the following issues be studied:

1. Animal infectivity should be used to measure the level of inactivation to provide a more relevant measurement.
2. Morphological examinations should be carried out with cysts treated with a wider range of oxidant doses. In addition, cysts treated with different CT combinations resulting in similar inactivation levels should be examined.
3. The study should be extended at the molecular level. Protein profile of both surface and cytoplasm should be studied for untreated control cysts and cysts treated with chemicals.
4. Permeability studies using different molecular size probes should be used to determine the exact damage caused by different chemicals at different levels of treatment.

6. LITERATURE CITED

Abel, E. S., Davids, B. J., Robles, L. D., Loflin, C. E., Gillin, F. D. and Chakrabarti, R. 2001: Possible roles of protein kinase A in cell motility and excystation of the early diverging eukaryote *Giardia lamblia*. Journal of Biological Chemistry **276**, 10320-10329.

Adam, R. D. 1991: The Biology of *Giardia* spp. Microbiological Reviews **55**, 706-732.

Adam, R. D. 2001: Biology of *Giardia lamblia*. Clinical Microbiology Reviews **14**, 447.

Aley, S. B. and Gillin, F. D. 1995: Specialized surface adaptations of *Giardia lamblia*. Infectious Agents and Disease **4**, 161-166.

Arstila, A. U. and Trump, B. F. 1969: Autophagocytosis: origin of membrane and hyolytic enzymes. Virchow Archives in Biological Cell Pathology **2**, 85-90.

Bablon, G. P. 1991: Practical Application of Ozone: Principles and Case Studies. In: Ozone in Water Treatment Application and Engineering. AWWARF.

Belosevic, M., Faubert, G. M., MacLean, J. D., Law, C. and Croll, N. A. 1983: *Giardia lamblia* Infections in Mongolian Gerbils: An Animal Model. Journal of Infectious Diseases **147**, 222-226.

Belosevic, M., Guy, R. A., Taghi-Kilani, R., Neumann, N. F., Gyürék, L. L., Liyanage, L. R. J., Millard, P. J. & Finch, G. R. 1997: Nucleic acid stains as indicators of *Cryptosporidium parvum* oocyst viability. International Journal for Parasitology **27**, 787-798.

Berman, D., Rice, E. W. and Hoff, J. C. 1988: Inactivation of Particle Associated Coliforms by Chlorine and Monochloramine. Applied and Environmental Microbiology **54**, 507-512.

Bernal, R. M., Tovar, R., Santos, J. I. and Munoz, M. D. 1998: Possible role of calmodulin in excystation of *Giardia lamblia*. Parasitology Research **84**, 687-693.

Bingham, A. K., Jarroll, E. L., Jr, Meyer, E. A. and Radulescu, S. 1979a: Induction of *Giardia* Excystation and the Effect of Temperature on Cyst Viability as Compared by Eosin-exclusion and *In vitro* Excystation. In: Waterborne Transmission of Giardiasis, Vol. EPA-600/9-79-001 (Jakubowski, W. and Hoff, J. C. eds). United States Environmental Protection Agency, Cincinnati, OH. pp. 217-229.

Bingham, A. K., Jarroll, E. L., Jr., Meyer, E. A. and Radulescu, S. 1979b: *Giardia* sp.: Physical Factors of Excystation *In vitro*, and Excystation vs Eosin Exclusion as Determinants of Viability. Experimental Parasitology **47**, 284-291.

Bingham, A. K. and Meyer, E. A. 1979: *Giardia* Excystation can be Induced *In vitro* in Acidic Solutions. Nature (London) **277**, 301-302.

Bockman, D. E. and Winborn, W. B. 1968: Electron microscopic localization of exogenous ferritin within vacuoles of *Giardia muris*. Journal of Protozoology **15**, 26-30.

Bourgine, F. P., Chapman, J. I., Kerai, H. and Green, J. G. 1993: Ozone and the Formation of Bromate in Water Treatment. Journal of the Institution of Water and Environmental Management **7**, 571-576.

Bradbury, J. S. 1998: Inactivation of *Giardia muris* Cysts Using Multiple Disinfectants. M. Sc., University of Alberta.

Bryant, E. A., Fulton, G. P. and Budd, G. C. 1992: Disinfection alternatives for safe drinking water. Van Nostrand Reinhold, New York.

Buchel, L. A., Chochillon, C., Gorenflot, A., Brugerolle, G., Gobert, J. G. and Savel, J. 1991a: *Giardia* from Humans - Analysis of *in vitro* Excystation by Transmission Electron-Microscopy. Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales **185**, 69-77.

Buchel, L. A., Gorenflot, A., Chochillon, C., Savel, J. and Gobert, J. G. 1987: *In vitro* excystation of *Giardia* from humans: a scanning electron microscopy study. Journal of Parasitology **73**, 487-493.

Bull, R. J., Birnbaum, L. S., Cantor, K. P., Rose, J. B., Butterworth, B. E., Regram, R. and Tuomisto, J. 1995: Water Chlorination: Essential Process or Cancer Hazard? Fundamental and Applied Toxicology **28**, 155-166.

Butterfield, C. T., Wattie, E., Megregian, S. and Chambers, C. W. 1943: Influence of pH and Temperature on the Survival of Coliforms and Enteric Pathogens When Exposed to Free Chlorine. Public Health Reports **58**, 1837-1866.

Campbell, I., Tzipori, S., Hutchison, G. and Angus, K. W. 1982: Effect of Disinfectants on Survival of *Cryptosporidium* Oocysts. Veterinary Record **111**, 414-415.

Campbell, J. D. and Faubert, G. M. 1994: Comparative studies on *Giardia lamblia* encystation *in vitro* and *in vivo*. Journal of Parasitology **80**, 36-44.

Chang, S. L. 1944: Destruction of Micro-Organisms. Journal of the American Water Works Association **36**, 1192-1207.

Chang, S. L. 1971: Modern Concept of Disinfection. Journal of the Sanitary Engineering Division, Proceedings of the American Society of Civil Engineering **97**, 689-707.

Chavez, B., Cedillo-Rivera, R. and Martinez-Palomo, A. 1992: *Giardia lamblia*: ultrastructural study of the *in vitro* effect of benzimidazoles. Journal of Protozoology **39**, 510-515.

Cheissin, E. M. 1964: Ultrastructure of *Lamblia duodenalis*. I. body surface, sucking disc, and median bodies. Journal of Protozoology **11**, 91-98.

Chen, Y. S. R., Sproul, O. J. and Rubin, A. J. 1985: Inactivation of *Naegleria gruberi* Cysts by Chlorine Dioxide. Water Research **19**, 783-789.

Chick, H. 1908: An Investigation of the Laws of Disinfection. Journal of Hygiene (Cambridge) **8**, 92-158.

Christensen, E. and Giese, A. 1954: Changes in adsorption spectra of nucleic acids and their derivatives following exposure to ozone and ultraviolet radiations. Archives in Biochemical Biophysics **51**, 208-216.

Churn, C. C., Bates, R. C. and Boardman, G. D. 1983: Mechanism of chlorine inactivation of DNA-containing parvovirus H-1. Applied and Environmental Microbiology **46**, 1394-1402.

Clark, R. M., Rice, E. W., Pierce, B. K., Johnson, C. H. and Fox, K. R. 1994: Effect of Aggregation on *Vibrio cholerae* Inactivation. Journal of Environmental Engineering **120**, 875-887.

Coggins, J. R. and Schaefer, F. W., III. 1984: *Giardia muris*: Scanning Electron Microscopy of *In Vitro* Excystation. Experimental Parasitology **57**, 62-67.

Coggins, J. R. and Schaefer, F. W., III. 1986: *Giardia muris*: Ultrastructural Analysis of *In Vitro* Excystation. Experimental Parasitology **61**, 219-228.

Craun, G. F. and Calderon, R. L. 1999: Waterborne Disease Outbreaks: Their Causes, Problems, and Challenges to Treatment Barriers. In: Waterborne Pathogens. American Water Works Association, Denver, CO. pp. 3-17.

Craun, G. F., Nwachuku, N., Calderon, R. L. and Craun, M. F. 2002: Outbreaks in drinking water systems, 1991-1998. Journal of Environmental Health **65**, 16-23.

Dave, S. A. 1999: Efficacy of ozone against *Salmonella enteritidis* in aqueous suspensions and on poultry meat, Ohio State University, Columbus, Ohio.

Degrémont. 1979: Water Treatment Handbook. Halsted Press, Paris, France.

deMik, G. and deGroot, I. 1977: Mechanisms of Inactivation of Bacteriophage Psi-X174 and Its DNA in Aerosols by Ozone and Ozonized Cyclohexene. Journal of Hygiene **78**, 199-211.

Dennis, W. H., Olivieri, V. P. and Kruse, C. W. 1979: Mechanism of Disinfection - Incorporation of Cl-36 into F2 Virus. Water Research **13**, 363-369.

deRegnier, D. P., Cole, L., Schupp, D. G. and Erlandsen, S. L. 1989: Viability of *Giardia* Cysts Suspended in Lake, River, and Tap Water. Applied and Environmental Microbiology **55**, 1223-1229.

Diamond, L. S., Harlow, D. and Cunnick, C. C. 1978: A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Transactions of the Royal Society of Tropical Medicine and Hygiene **72**, 431-432.

Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. L., Milner, A. E. and Wyllie, A. H. 1992: Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochimica et a Biophysica Acta **1133**, 275-285.

Doré, M., Brunet, R., Legube, B. and Croue, J. P. 1987: The Role of Alkalinity in the Effectiveness of Processes of Oxidation by Ozone. In: The Role of Ozone in Water and Wastewater Treatment. Proceedings of the Second International Conference (Smith, D. W. and Finch, G. R. eds). TekTran International, Kitchener, Ontario. pp. 1-14.

Doroszkiewicz, W., Sikorska, I. and Jankowski, S. 1994: Studies on the Influence of Ozone on Complement Mediated Killing of Bacteria. Fems Immunology and Medical Microbiology **9**, 281-285.

Erlandsen, S. L., Bemrick, W. J. and Pawley, J. 1989: High-resolution electron microscopic evidence for the filamentous structure of the cyst wall in *Giardia muris* and *Giardia duodenalis*. Journal of Parasitology **75**, 787-797.

Erlandsen, S. L., Bemrick, W. J., Schupp, D. E., Shields, J. M., Jarroll, E. L., Sauch, J. F. and Pawley, J. B. 1990: High resolution immunogold localization of *Giardia* cyst wall antigens using field emission SEM with secondary and backscatter electron imaging. Journal of Histochemistry and Cytochemistry **38**, 625-632.

Erlandsen, S. L. and Chase, D. G. 1974: Morphological alterations in the microvillous border of villous epithelial cells produced by intestinal microorganisms. American Journal of Clinical Nutrition **27**, 1277-1286.

Fair, G. M., Morris, J. C., Chang, S. L., Weil, I. and Burden, R. P. 1948: The Behavior of Chlorine as a Water Disinfectant. Journal of the American Water Works Association **40**, 1051-1061.

Farooq, S., Chian, E. S. K. and Engelbrecht, R. S. 1977a: Basic Concepts in Disinfection with Ozone. Journal of the Water Pollution Control Federation **49**, 1818-1831.

Farooq, S., Englebrecht, R. S. and Chian, E. S. K. 1977b: Influence of Temperature and U. V. Light on Disinfection With Ozone. Water Research **11**, 737-741.

Faubert, G., Reiner, D. S. and Gillin, F. D. 1991: *Giardia lamblia* - Regulation of Secretory Vesicle Formation and Loss of Ability to Reattach During Encystation *in vitro*. Experimental Parasitology **72**, 345-354.

Fauris, C., Danglot, C. and Vilagines, R. 1982: Parameters Influencing Poliovirus Inactivation by Chlorine. Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences **295**, 73-76.

Feely, D. E. 1979: Functional-Morphology of *Giardia* Trophozoites Isolated from Rat Small-Intestine. Anatomical Record **193**, 535-535.

Feely, D. E. 1986: A Simplified Method for *In vitro* Excystation of *Giardia muris*. Journal of Parasitology **72**, 474-475.

Feely, D. E. 1988: Morphology of the Cyst of *Giardia microti* by Light and Electron-Microscopy. Journal of Protozoology **35**, 52-54.

Feely, D. E. and Dyer, J. K. 1987: Localization of Acid Phosphatase Activity in *Giardia lamblia* and *Giardia muris* Trophozoites. Journal of Protozoology **34**, 80-83.

Feely, D. E. and Erlandsen, S. L. 1981: *in vitro* Analysis of the Attachment of *Giardia* Trophozoites. Journal of the American Osteopathic Association **80**, 781-781.

Feely, D. E. and Erlandsen, S. L. 1982: Effect of Cytochalasin-B, Low Ca++ Concentration, Iodoacetic Acid, and Quinacrine-Hcl on the Attachment of *Giardia* Trophozoites *in vitro*. Journal of Parasitology **68**, 869-873.

Feely, D. E. and Erlandsen, S. L. 1985: Morphology of *Giardia agilis* - Observation by Scanning Electron-Microscopy and Interference Reflection Microscopy. Journal of Protozoology **32**, 691-693.

Feely, D. E., Erlandsen, S. L. and Schoolmeyer, J. V. 1980: Morphology of Isolated *Giardia* Trophozoites. Journal of the American Osteopathic Association **80**, 225-225.

Feely, D. E., Gardner, M. D. and Hardin, E. L. 1991: Excystation of *Giardia muris* Induced by a Phosphate Bicarbonate Medium - Localization of Acid Phosphatase. Journal of Parasitology **77**, 441-448.

Feely, D. E., Holberton, D. V. and Erlandsen, S. L. 1990: The Biology of *Giardia*. In: Giardiasis, Vol. 3 (Meyer, E. A. ed). Elsevier, Amsterdam. pp. 11-49.

Feely, D. E., Schollmeyer, J. V. and Erlandsen, S. L. 1982: *Giardia* Spp - Distribution of Contractile Proteins in the Attachment Organelle. Experimental Parasitology **53**, 145-154.

Filice, F. P. 1952: Studies on the Cytology and Life History of a *Giardia* from the Laboratory Rat. University of California Publications in Zoology **57**, 53-146.

Finch, G. R. and Black, E. K. 1994: Chemical Inactivation of *Cryptosporidium parvum* in Drinking Water. In: Critical Issues in Water and Wastewater Treatment, Proceedings of the 1994 National Conference on Environmental Engineering (Ryan, J. N. and Edwards, M. eds). American Society of Civil Engineers, New York, NY. pp. 42-49.

Finch, G. R., Black, E. K., Gyürék, L. and Belosevic, M. 1993a: Ozone inactivation of *Cryptosporidium parvum* in demand-free phosphate buffer determined by *in vitro* excystation and animal infectivity. Applied and Environmental Microbiology **59**, 4203-4210.

Finch, G. R., Black, E. K., Gyürék, L. and Belosevic, M. 1994: Ozone Disinfection of *Giardia* and *Cryptosporidium*. AWWA Research Foundation and American Water Works Association, Denver, CO.

Finch, G. R., Black, E. K., Labatiuk, C. W., Gyürék, L. and Belosevic, M. 1993b: Comparison of *Giardia lamblia* and *Giardia muris* Cyst Inactivation by Ozone. Applied and Environmental Microbiology **59**, 3674-3680.

Finch, G. R. and Li, H. 1999: Inactivation of *Cryptosporidium* at 1°C using Ozone or Chlorine Dioxide. Ozone: Science and Engineering **21**, 477-486.

Finch, G. R., Li, H., Lewin, N. and Belosevic, M. 2000a: Sequential Disinfection Design Criteria for Inactivation of *Cryptosporidium parvum* in Drinking Water (submitted for review). AWWA Research Foundation and American Water Works Association, Denver, CO.

Finch, G. R., Liyanage, L. R. J., Bradbury, J. S., Gyürék, L. L. and Belosevic, M. 2000b: Synergistic Effects of Multiple Disinfectants. (in press). AWWA Research Foundation and American Water Works Association, Denver, CO.

Finch, G. R., Yuen, W. C. and Uibel, B. J. 1992: Inactivation of Escherichia-Coli Using Ozone and Ozone - Hydrogen-Peroxide. Environmental Technology **13**, 571-578.

Foegeding, P. M. 1983: Bacterial Spore Resistance to Chlorine Compounds. Food Technology **37**, 100-and.

Foegeding, P. M. 1985: Ozone inactivation of *Bacillus* and *Clostridium* spore population and the importance of the spore coat to resistance. Food Microbiology **2**, 123-134.

Forni, L., Bahnemann, D. and Hart, E. J. 1982: Mechanism of the Hydroxide Ion Initiated Decomposition of Ozone in Aqueous Solution. Journal of Physical Chemistry **86**, 255-259.

Friend, D. S. 1966: The fine structure of *Giardia muris*. Journal of Cell Biology **29**, 317-332.

Gillin, F. D. 1987: *Giardia lamblia* - the Role of Conjugated and Unconjugated Bile-Salts in Killing by Human-Milk. Experimental Parasitology **63**, 74-83.

Gillin, F. D., Boucher, S. E. and Reiner, D. S. 1987: Stimulation of *in vitro* Encystation of *Giardia lamblia* by Small Intestinal Conditions. Clinical Research **35**, A475-A475.

Gillin, F. D., Boucher, S. E., Rossi, S. S. and Reiner, D. S. 1989: *Giardia lamblia* - the Roles of Bile, Lactic-Acid, and Ph in the Completion of the Life-Cycle *in vitro*. Experimental Parasitology **69**, 164-174.

Gillin, F. D., Gault, M. J., Hofmann, A. F., Gurantz, D. and Sauch, J. F. 1986: Biliary Lipids Support Serum-Free Growth of *Giardia lamblia*. Infection and Immunity **53**, 641-645.

Gillin, F. D. and Reiner, D. S. 1982: Attachment of the Flagellate *Giardia lamblia* - Role of Reducing Agents, Serum, Temperature, and Ionic Composition. Molecular and Cellular Biology **2**, 369-377.

Gillin, F. D., Reiner, D. S. and Boucher, S. E. 1988: Small-Intestinal Factors Promote Encystation of *Giardia lamblia in vitro*. Infection and Immunity **56**, 705-707.

Gillin, F. D., Reiner, D. S. and McCaffery, J. M. 1996: Cell Biology Of the Primitive Eukaryote *Giardia lamblia*. Annual Review of Microbiology **50**, 679-705.

Gillin, F. D., Reiner, D. S. and McCaffery, M. 1991: Organelles of Protein Transport in *Giardia lamblia*. Parasitology Today **7**, 113-116.

Ginn, F. L., Hochstein, P. and Trump, B. F. 1969: Membrane alterations in hemolysis: Internalization of plasmalemma induced by primaquine. Science **164**, 843-845.

Glaze, W. H. 1986: Reaction Products of Ozone: A Review. Environmental Health Perspectives **69**, 151-157.

Glaze, W. H., Kang, J. W. and Chapin, D. H. 1987: The Chemistry of Water Treatment Processes Involving Ozone, Hydrogen Peroxide, and Ultraviolet Radiation. Ozone: Science and Engineering **9**, 335-352.

Goldstein, B. D. and McDonagh, E. M. 1975: Effect of Ozone on Cell Membrane Protein Fluorescence I. *in vitro* Studies Utilizing the Red Cell Membrane. Environmental Resources **9**, 179-186.

Graham, N. J. D., Buckley, D. and Croll, B. 1992: Alternative Disinfection Regimes for Trihalomethane Control - Significance of Pre-Disinfectant Dose. Environmental Technology **13**, 461-471.

Greenberg, A. E., Clesceri, L. S. and Eaton, A. D. (1992): Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC.

Gurol, M. D. and Singer, P. C. 1982: Kinetics of Ozone Decomposition: A Dynamic Approach. Environmental Science and Technology **16**, 377-383.

Gyürék, L. L., Finch, G. R., Liyanage, L. R. J. and Belosevic, M. 1996: Disinfection Requirements for Single and Sequential Inactivation of *Cryptosporidium* Using Chlorine Species. In: 4th Environmental Engineering Specialty Conference. Canadian Society for Civil Engineering, Montréal, Quebec. pp. 29-40.

Gyürék, L. L., Li, H., Belosevic, M. and Finch, G. R. 1999: Ozone Inactivation Kinetics of *Cryptosporidium parvum* in Phosphate Buffer. Journal of Environmental Engineering **125**, 913-924.

Gyürék, L. L., Neumann, N. F., Finch, G. R. and Belosevic, M. 2001: Comparison Between Animal Infectivity and Nucleic Acid Staining for Determination of Viability of Ozone-Inactivated *Cryptosporidium parvum* Oocysts. Ozone: Science and Engineering **23**, 1-13.

Haas, C. N. and Engelbrecht, R. S. 1980: Physiological alterations of vegetative microorganisms resulting form chlorination. Journal - Water Pollution Control Federation **52**, 1976-1989.

Hall, R. M. and Sobsey, M. D. 1993: Inactivation of Hepatitis A Virus And MS2 By Ozone And Ozone-Hydrogen Peroxide In Buffered Water. Water Science and Technology **27**, 371-378.

Hart, E. J., Sehested, K. and Holcman, J. 1983: Molar Absorptivities of Ultraviolet and Visible Bands of Ozone in Aqueous Solutions. Analytical Chemistry **55**, 46-49.

Healy, G. R. 1979: The Presence and Absence of *Giardia lamblia* in Studies on Parasite Prevalence in the U.S.A. In: Waterborne Transmission of Giardiasis, Vol. EPA-600/9-79-001 (Jakubowski, W. and Hoff, J. C. eds). United States Environmental Protection Agency, Cincinnati, OH. pp. 92-103.

Helmer, R. D. and Finch, G. R. 1993: Use of MS2 Coliphage as a Surrogate for Enteric Viruses in Surface Waters Disinfected with Ozone. Ozone: Science and Engineering **15**, 279-293.

Herbold, K., Flehmig, B. and Botzenhart, K. 1989: Comparison of Ozone Inactivation, in Flowing Water, of Hepatitis A Virus, Poliovirus 1, and Indicator Organisms. Applied and Environmental Microbiology **55**, 2949-2953.

Herson, D. S., McGonigle, B., Payer, M. A. and Baker, K. H. 1987: Attachment as a Factor in the Protection of Enterobacter cloacae from Chlorination. Applied and Environmental Microbiology **53**, 1178-1180.

Hewes, C. G. and Davison, R. R. 1971: Kinetics of Ozone Decomposition and Reaction with Organics in Water. AIChE J **17**, 141-147.

Hibler, C. P., Hancock, C. M., Perger, L. M., Wegrzyn, J. C. and Swabby, K. D. 1987: Inactivation of *Giardia* Cysts with Chlorine at 0.5°C to 5.0°C. American Water Works Association, Denver, CO.

Hoigné. 1988: The Chemistry of Ozone in Water. In: Process Technologies for Water Treatment (Stucki, S. ed). Plenum Press, New York, NY. pp. 121-143.

Hoigné, J. and Bader, H. 1978: Ozone Initiated Oxidations of Solutes in Wastewater: A Reaction Kinetic Approach. Progress in Water Technology **10**, 657-671.

Hoigné, J. and Bader, H. 1979: Ozonation of Water: Selectivity and Rate of Oxidation of Solutes. Ozone: Science and Engineering **1**, 73-85.

Hoigné, J. and Bader, H. 1983: Rate Constants of Reactions of Ozone with Organic and Inorganic Compounds in Water - II. Dissociating Organic Compounds. Water Research **17**, 185-194.

Hoigné, J. and Bader, H. 1994: Characterization of Water Quality Criteria for Ozonation Processes. Part II: Lifetime of Added Ozone. Ozone: Science and Engineering **16**, 121-134.

Hoigné, J., Bader, H., Haag, W. R. and Staehelin, J. 1985: Rate Constants of Reactions of Ozone with Organic and Inorganic Compounds in Water - III. Inorganic Compounds and Radicals. Water Research **19**, 993-1004.

Holberton, D. V. 1973: Fine structure of the ventral disk apparatus and the mechanism of attachment in the flagellate *Giardia muris*. Journal of Cellular Science **13**, 11-41.

Holberton, D. V. 1981: Arrangement of subunits in microribbons from *Giardia*. Journal of Cell Science **47**, 165-185.

Ignatenko, A. V. and Cherenkevich, S. N. 1985: Covalent cross-linking and degradation of proteins caused by ozone. Biofizika **30**, 18-22.

Ingram, M. and Barnes, E. M. 1949: Sterilization by Means of Ozone. Journal of Applied Bacteriology **17**, 246-271.

Jacangelo, J. G., Olivieri, V. P. and Kawata, K. 1991: Investigating the Mechanism of Inactivation of *Escherichia coli* B by Monochloramine. Journal of the American Water Works Association **83**, 80-87.

Janoff, E. N. and Smith, P. D. 1990: The role of immunity in *Giardia* infections. In: Giardiasis (Meyer, E. A. ed). Elsevier, Amsterdam. pp. 215-234.

Jarroll, E. L., Jr, Bingham, A. K. and Meyer, E. A. 1981a: Effect of Chlorine on *Giardia lamblia* Cyst Viability. Applied and Environmental Microbiology **41**, 483-487.

Jarroll, E. L., Jr. 1988: Effect of Disinfectants on *Giardia* Cysts. CRC Critical Reviews in Environmental Control **18**, 1-28.

Jarroll, E. L., Manning, P., Berrada, A., Hare, D. and Lindmark, D. G. 1989a: Biochemistry and Metabolism of *Giardia*. Journal of Protozoology **36**, 190-197.

Jarroll, E. L., Manning, P., Lindmark, D. G., Coggins, J. R. and Erlandsen, S. L. 1989b: *Giardia* Cyst Wall-Specific Carbohydrate - Evidence for the Presence of Galactosamine. Molecular and Biochemical Parasitology **32**, 121-131.

Jarroll, E. L., Muller, P. J., Meyer, E. A. and Morse, S. A. 1981b: Lipid and carbohydrate metabolism of *Giardia lamblia*. Molecular and Biochemical Parasitology **2**, 187-196.

Jones, K. H. and Senft, J. A. 1985: An Improved Method to Determine Cell Viability by Simultaneous Staining with Fluorescein Diacetate-Propidium Iodide. The Journal of Histochemistry and Cytochemistry **33**, 77-79.

Kabnick, K. S. and Peattie, D. A. 1990: Insitu Analyses Reveal That the 2 Nuclei of *Giardia lamblia* Are Equivalent. Journal of Cell Science **95**, 353-360.

Kane, A. V., Ward, H. D., Keusch, G. T. and Pereira, M. 1991: *In Vitro* Encystation of *Giardia lamblia* - Large Scale Production of *In vitro* Cysts and Strain and Clone Differences in Encystation Efficiency. Journal of Parasitology **77**, 974-981.

Kappus, K. D., Jr., R. G. L., Juranek, D. D., Roberts, J. M. and Spencer, H. C. 1994: Intestinal Parasitism in the United States: Update on a Continuing Problem. American Journal of Tropical Medicine and Hygiene **50**, 705-713.

Karazewski, B. 1994: How to use chlorine as a disinfectant. Water Technology **17**, 28-30.

Katelaris, P. H., Naeem, A. and Farthing, M. J. 1994: Activity of metronidazole, azithromycin and three benzimidazoles on *Giardia lamblia* growth and attachment to a human intestinal cell line. Alimentary Pharmacology and Therapeutics **8**, 187-192.

Kattenbach, W. M., Pimenta, P. F., de Souza, W. and Pinto da Silva, P. 1991: *Giardia duodenalis*: a freeze-fracture, fracture-flip and cytochemistry study. Parasitology Research **77**, 651-658.

Katz, A., Narkis, N., Orshansky, F., Friedland, E. and Kott, Y. 1994: Disinfection of Effluent by Combinations of Equal Doses of Chlorine Dioxide and Chlorine Added Simultaneously Over Varying Contact Times. Water Research **28**, 2133-2138.

Katzenelson, E., Koerner, G., Biedermann, N., Peleg, M. and Shuval, H. I. 1979: Measurement of the Inactivation Kinetics of Poliovirus by Ozone in a Fast Flow Mixer. Applied and Environmental Microbiology **37**, 715-718.

Khadre, M. A. and Yousef, A. E. 2001: Sporicidal action of ozone and hydrogen peroxide: a comparative study. International Journal of Food Microbiology **71**, 131-138.

Kim, C. K., Gentile, D. M. and Sproul, O. J. 1980: Mechanism of Ozone Inactivation of Bacteriophage-F2. Applied and Environmental Microbiology **39**, 210-218.

Kim, C. K., Kim, C. H. and Mheen, T. I. 1984: Destructive action of ozone on the capsid and deoxyribinuclei acid of bacteriophage T4. Sanop Misaengmul Hakhoechi **12**, 105-109.

Knodler, L. A., Noiva, R., Mehta, K., McCaffery, J. M., Aley, S. B., Svard, S. G., Nystul, T. G., Reiner, D. S., Silberman, J. D. and Gillin, F. D. 1999: Novel protein-disulfide isomerases from the early-diverging protist *Giardia lamblia*. Journal of Biological Chemistry **274**, 29805-29811.

Knox, W. E. and Stumpf, P. K. 1948: The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. Journal of Bacteriology **55**, 451-458.

Ko, Y. W., Chiang, P. C. and Chang, E. E. 1996: The Effect of Bromide Ion on the Formation of Organohalogen Disinfection By-Products During Ozonation. Ozone: Science and Engineering **18**, 349-361.

Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A. and Sterling, C. R. 1990: Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on *Cryptosporidium parvum* Oocyst Viability. Applied and Environmental Microbiology **56**, 1423-1428.

Kott, Y., Vinokur, L. and Ben-Ari, H. 1980: Combined Effects of Disinfectants on Bacteria and Viruses. In: Water Chlorination: Environmental Impact and Health Effects, Vol. 3 (Jolley, R. L., Brungs, W. A. and Cumming, R. B. eds). Ann Arbor Science Publishers, Ann Arbor, Mich. pp. 677-686.

Kouame, Y. and Haas, C. N. 1991: Inactivation of *E. coli* by Combined Action of Free Chlorine and Monochloramine. Water Research **25**, 1027-1032.

Kulikovsky, A., Pankratz, H. S. and Sadoff, H. L. 1975: Ultrastructural and Chemical Changes in Spores of *Bacillus cereus* after Action of Disinfectants. Journal of Applied Bacteriology **38**, 39-and.

Lanfredi-Rangel, A., Attias, M., de Carvalho, T. M. U., Kattenbach, W. M. and de Souza, W. 1998: The peripheral vesicles of trophozoites of the primitive protozoan *Giardia lamblia* may correspond to early and late endosomes and to lysosomes. Journal of Structural Biology **123**, 225-235.

Lanfredi-Rangel, A., Kattenbach, W. M., Diniz, J. A. and de Souza, W. 1999: Trophozoites of *Giardia lamblia* may have a Golgi-like structure. FEMS Microbiology Letters **181**, 245-251.

Langlais, B. and Perrine, D. 1986: Action of Ozone on Trophozoites and Free Ameba Cysts, Whether Pathogenic or Not. Ozone-Science and Engineering **8**, 187-198.

Langlais, B., Perrine, D., Joret, J. C. and Chenu, J. P. 1990: The C.T Value Concept for Evaluation of Disinfection Process Efficiency; Particular Case of Ozonation for Inactivation of Some Protozoan: Free Living Amoeba and *Cryptosporidium*. In: New Developments: Ozone in Water and Wastewater Treatment. Proceedings of the International Ozone Association Spring Conference, March 27-29 1990, Shreveport, LO. International Ozone Association, Pan American Committee, Norwalk, CT.

Langlais, B., Reckhow, D. A. and Brink, D. R. (1991): Ozone in Water Treatment: Application and Engineering, Lewis Publishers, Inc., Chelsea, MI.

Leahy, J. G., Rubin, A. J. and Sproul, O. J. 1987: Inactivation of *Giardia muris* Cysts by Free Chlorine. Applied and Environmental Microbiology **53**, 1448-1453.

Lee, J. and Deininger, R. A. 2000: Survival of bacteria after ozonation. Ozone-Science and Engineering **22**, 65-75.

Lengerich, E. J., Addiss, D. G. and Juranek, D. D. 1994: Severe Giardiasis in the United States. Clinical Infectious Diseases **18**, 760-763.

Levine, N. D. 1979: *Giardia lamblia*: Classification, Structure, Identification. In: Waterborne Transmission of Giardiasis, Vol. EPA-600/9-79-001 (Jakubowski, W. and Hoff, J. C. eds). United States Environmental Protection Agency, Cincinnati, OH. pp. 2-8.

Li, H., Finch, G. R. and Belosevic, M. 1999: Effect of Water Temperature on the Sequential Inactivation of *Cryptosporidium* Using Ozone Followed by Free Chlorine. Paper pres. American Water Works Association Water Quality and Technology Conference, Tampa, FL.

Li, H., Finch, G. R., Smith, D. W. and Belosevic , M. 2001a: Sequential Disinfection Design Criteria for Inactivation of *Cryptosporidium parvum* Oocysts in Drinking Water. AWWA Research Foundation and the American Water Works Association, Denver, CO.

Li, H., Finch, G. R., Smith, D. W. and Belosevic, M. 2001b: Sequential Inactivation of *Cryptosporidium parvum* Using Ozone and Chlorine. Water Research.

Lin, S. D. 1985: *Giardia lamblia* and Water Supply. Journal of the American Water Works Association **77**, 40-47.

Lindmark, D. G. 1988: *Giardia lamblia* - Localization of Hydrolase Activities in Lysosome-Like Organelles of Trophozoites. Experimental Parasitology **65**, 141-147.

Liu, O. C. 1971: Relative resistance of twenty human enteric viruses to free chlorine. Paper pres. thirteenth Water Quality Conference, University of Illinois, Urbana-Champaign.

Liyanage, L. R. J., Finch, G. R. and Belosevic, M. 1997a: Sequential Disinfection of *Cryptosporidium parvum* by Ozone and Chlorine Dioxide. Ozone: Science and Engineering **19**, 409-423.

-. 1997b: Synergistic Effects of Sequential Exposure of *Cryptosporidium* Oocysts to Chemical Disinfectants. In: Proceedings of International Symposium on Waterborne *Cryptosporidium*, Newport Beach, CA. March 2-5, 1997 (Fricker, C. R., Clancy, J. L. and Rochelle, P. A. eds). American Water Works Association, Denver, CO.

Liyanage, L. R. J., Gyürék, L. L., Belosevic, M. and Finch, G. R. 1997c: Effect of Chlorine Dioxide Preconditioning on Inactivation of *Cryptosporidium* by Free Chlorine and Monochloramine: Process Design Requirements. In: Proceedings Water Quality Technology Conference, November 17-21, 1996, Boston, MA. American Water Works Association, Denver, CO.

Luchtel, D. L., Lawrence, W. P. and DeWalle, F. B. 1980: Electron microscopy of *Giardia lamblia* cysts. Applied and Environmental Microbiology **40**, 821-832.

Lujan, H. D., Mowatt, M. R., Conrad, J. T. and Nash, T. E. 1996: Increased expression of the molecular chaperone BiP/GRP78 during the differentiation of a primitive eukaryote. Biology of the Cell **86**, 11-18.

Lujan, H. D., Mowatt, M. R. and Nash, T. E. 1997: Mechanisms of *Giardia lamblia* differentiation into cysts. Microbiology and Molecular Biology Reviews **61**, 294-and.

Lykins, B. W., Jr., Koffskey, W. E. and Patterson, K. S. 1994: Alternative Disinfectants for Drinking Water Treatment. Journal of Environmental Engineering **120**, 745-758.

Majewska, A. C., Kasprzak, W. and Kaczmarek, E. 1993: Comparative Morphometry of *Giardia* Trophozoites from Man and Animals. Acta Protozoologica **32**, 191-197.

Manning, P., Erlandsen, S. L. and Jarroll, E. L. 1992: Carbohydrate and amino acid analyses of *Giardia muris* cysts. Journal of Protozoology **39**, 290-296.

Maris, P. 1995: Modes of action of disinfectants. Revue Scientifique et Technique **14**, 47-55.

McCaffery, J. M., Faubert, G. M. and Gillin, F. D. 1994: *Giardia lamblia* - Traffic of a Trophozoite Variant Surface Protein and a Major Cyst Wall Epitope During Growth, Encystation, and Antigenic Switching. Experimental Parasitology **79**, 236-249.

McCaffery, J. M. and Gillin, F. D. 1994: *Giardia lamblia* - Ultrastructural Basis of Protein-Transport During Growth and Encystation. Experimental Parasitology **79**, 220-235.

McKerrow, J. H., Sun, E., Rosenthal, P. J. and Bouvier, J. 1993: The Proteases and Pathogenicity of Parasitic Protozoa. Annual Review of Microbiology **47**, 821-853.

McKersie, B. D., Hucl, P. and Beversdorf, W. D. 1982: Solute leakage from susceptible and tolerant cultivars of *Phaseolus vulgaris* following ozone exposure. Canadian Journal of Botany **60**, 73-78.

Meng, T. C., Aley, S. B., Svard, S. G., Smith, M. W., Huang, B., Kim, J. and Gillin, F. D. 1996a: Immunolocalization and Sequence Of Caltractin/Centrin From the Early Branching Eukaryote *Giardia lamblia*. Molecular And Biochemical Parasitology. Jul **79**, 103-108.

Meng, T. C., Hetsko, M. L. and Gillin, F. D. 1996b: Inhibition Of *Giardia lamblia* Excystation By Antibodies Against Cyst Walls and By Wheat Germ Agglutinin. Infection And Immunity. **64**, 2151-2157.

Meng, T. C., Hetsko, M. L., Reiner, D. S. and Gillin, F. D. 1994: Antibodies against the *Giardia lamblia* Cyst Wall Inhibit Excystation *in vitro*. Faseb Journal **8**, A491-A491.

Meyer, E. A. 1990: Taxonomy and Nomenclature. In: Giardiasis, Vol. 3 (Meyer, E. A. ed). Elsevier, Amsterdam. pp. 51-60.

Morgan, U. M., Reynoldson, J. A. and Thompson, R. C. A. 1993: Activities of Several Benzimidazoles and Tubulin Inhibitors against *Giardia* Spp *in vitro*. Antimicrobial Agents and Chemotherapy **37**, 328-331.

Mudd, J. B., Leavitt, R., Ongun, A. and McManus, T. T. 1969: Reaction of ozone with amino acids and protein. Atmosphere Environment **3**, 669-682.

Murray, R. G., Pumela, S. and E., E. H. 1965: The location of the mucopeptide of sections of the cell wall of *Escherichia coli* and other gram negative bacteria. Canadian Journal of Microbiology **11**, 547-560.

Narcisi, E. M., Glover, C. V. C. and Fechheimer, M. 1998: Fibrillarin, a conserved pre-ribosomal RNA processing protein of *Giardia*. Journal of Eukaryotic Microbiology **45**, 105-111.

Narcisi, E. M., Paulin, J. J. and Fechheimer, M. 1994: Presence and localization of vinculin in *Giardia*. Journal of Parasitology **80**, 468-473.

Neumann, N. F., Gyürék, L. L., Gammie, L., Finch, G. R. and Belosevic, M. 2000: Comparison of Animal Infectivity and Nucleic Acid Staining for Assessment of *Cryptosporidium parvum* Viability in Water. Applied and Environmental Microbiology **66**, 406-412.

Neuwirth, M., Roach, P. D., Buchanan Mappin, J. M., Wallis, P. M., Wallis, P. M. and Hammond, B. R. 1988: Effects of chlorine on the ultrastructure of *Giardia* cysts. Advances in *Giardia* research 133-135; Papers from the Calgary *Giardia* Conference held February 23-25, 1987, Calgary, Alberta, Canada.

Noss, C. I. and Olivieri, V. P. 1985: Disinfecting Capabilities of Oxychlorine Compounds. Applied and Environmental Microbiology **50**, 1162-1164.

O'Brien, R. T. and Newman, J. 1979: Structural and Compositional Changes Associated with Chlorine Inactivation of Polio-Viruses. Applied and Environmental Microbiology **38**, 1034-1039.

Peattie, D. A., Alonso, R. A., Hein, A. and Caulfield, J. P. 1989: Ultrastructural localization of giardins to the edges of disk microribbons of *Giardia lamblia* and the nucleotide and deduced protein sequence of alpha giardin. Journal of Cell Biology **109**, 2323-2335.

Perchorowicz, J. T. and Ting, I. P. 1974: Ozone effects on plant cell permeability. American Journal of Botany **61**, 787-793.

Perez, R. R., Nunez, S. A., Baluja, C. and Otero, M. L. 1995: Ozonation kinetics of glucosamine and N-acetyl glucosamine in aqueous medium. Ozone Science and Engineering **17**, 463-467.

Perrine, D., Barbier, D., Georges, P. and Langlais, B. 1984: The Destruction by Ozone of Amoeba Cysts in Recycled Swimming Pool Water. Effluent and Water Treatment Journal, 299-303.

Pryor, W. A., Dooley, M. M. and Church, D. F. 1983: Mechanisms for the Reaction of Ozone with Biological Molecules: The Source of the Toxic Effects of Ozone. In: Advances in Modern Environmental Toxicology (Mustafa, M. G. and Mehlman, M. A. eds). Ann Arbor Science Publishers, Ann Arbor, MI. pp. 7-19.

Radchenko, A. I. and Beier, T. V. 1995: [An electron microscopic study of the natural death of cyst cells in *Sarcosporidia*. II. Ultrastructural changes in the nuclei of cyst cells of *Sarcocystis muris*]. [Russian]. Tsitologiia **37**, 533-536, inside back cover.

Radchenko, A. I., Beier, T. V. and Gaibova, G. D. 1995: [An electron microscopic study of the natural death of cyst cells in Sarcosporidia. I. Ultrastructural changes in the cytoplasm of the cyst cells of Sarcocystis *muris* and *S. bovifelis*]. [Russian]. Tsitologiia **37**, 525-532.

Ransome, M. E., Whitmore, T. N. and Carrington, E. G. 1993: Effect of Disinfectants on the Viability of *Cryptosporidium parvum* Oocysts. Water Supply **11**, 75-89.

Reduker, D. W. and Speer, C. A. 1985: Factors Influencing Excystation in *Cryptosporidium* Oocysts from Cattle. Journal of Parasitology **71**, 112-115.

Reiner, D. S., Douglas, H. and Gillin, F. D. 1989: Identification and localization of cyst-specific antigens of *Giardia lamblia*. Infection and Immunity **57**, 963-968.

Reiner, D. S., McCaffery, J. M. and Gillin, F. D. 2001: Reversible interruption of *Giardia lamblia* cyst wall protein transport in a novel regulated secretory pathway. Cellular Microbiology **3**, 459-472.

Reiner, D. S., McCaffery, M. and Gillin, F. D. 1990: Sorting of cyst wall proteins to a regulated secretory pathway during differentiation of the primitive eukaryote, *Giardia lamblia*. European Journal of Cell Biology **53**, 142-153.

Rice, E. W., Hoff, J. C. and Schaefer, F. W., III. 1982: Inactivation of *Giardia lamblia* Cysts by Chlorine. Applied and Environmental Microbiology **43**, 250-251.

Rice, E. W. and Schaefer, F. W. d. 1981: Improved *in vitro* excystation procedure for *Giardia lamblia* cysts. Journal of Clinical Microbiology **14**, 709-710.

Rice, R. G. 1991: Recent Status of Ozone for Water Treatment in the United States. In: Chemistry for the Protection of the Environment, Environmental Science Research (Pawlowski, L., Lacy, W. J. and Dlugosz, J. J. eds). Plenum Publishing Corp., New York, NY. pp. 713-730.

Riesser, V. N., Perrich, J. R., Silver, E. B. and McCammon, J. R. 1977: Possible Mechanisms for Poliovirus Inactivation by Ozone. In: Forum on Ozone Disinfection (Fochtman, E. G., Rice, R. G. and Browning, M. F. eds). International Ozone Institute, Cleveland, OH. pp. 186-192.

Roberts-Thomson, I. C., Stevens, D. P., Mahmoud, A. A. F. and Warren, K. S. 1976: Giardiasis in the Mouse: An Animal Model. Gastroenterology **71**, 57-61.

Roy, D., Wong, P. K., Engelbrecht, R. S. and Chian, E. S. 1981: Mechanism of enteroviral inactivation by ozone. Applied and Environmental Microbiology **41**, 718-723.

Rubin, A. J., Evers, D. P., Eyman, C. M. and Jarroll, E. L. 1989: Inactivation of Gerbil-Cultured *Giardia lamblia* Cysts by Free Chlorine. Applied and Environmental Microbiology **55**, 2592-2594.

Sauch, J. F. and Berman, D. 1991: Immunofluorescence and Morphology of *Giardia lamblia* Cysts Exposed to Chlorine. Applied and Environmental Microbiology **57**, 1573-1575.

Schaefer, F. W., III. 1990: Methods for Excystation of *Giardia*. In: Giardiasis, Vol. 3 (Meyer, E. A. ed). Elsevier, Amsterdam. pp. 111-136.

Schaefer, F. W. d., Rice, E. W. and Hoff, J. C. 1984: Factors promoting *in vitro* excystation of *Giardia muris* cysts. Transactions of the Royal Society of Tropical Medicine and Hygiene **78**, 795-800.

Schupp, D. G. and Erlandsen, S. L. 1987: A New Method to Determine *Giardia* Cyst Viability: Correlation of Fluorescein Diacetate and Propidium Iodide Staining with Animal Infectivity. Applied and Environmental Microbiology **53**, 704-707.

Schupp, D. G., Januschka, M. M. and Erlandsen, S. L. 1988a: Morphology of *Giardia* Encystation *In vitro*. In: Advances in *Giardia* Research (Wallis, P. M. and Hammond, B. R. eds). The University of Calgary Press, Calgary, Alberta. pp. 29-32.

Schupp, D. G., Januschka, M. M., Sherlock, L. A. F., Stibbs, H. H., Meyer, E. A., Bemrick, W. J. and Erlandsen, S. L. 1988b: Production of Viable *Giardia* Cysts *in vitro* - Determination by Fluorogenic Dye Staining, Excystation, and Animal Infectivity in the Mouse and Mongolian Gerbil. Gastroenterology **95**, 1-10.

Scott, D. B. M. and Lesher, E. C. 1963: Effect of Ozone on Survival and Permeability of *Escherichia coli*. Journal of Bacteriology **85**, 567-576.

Sheffield, H. G. and Bjorvatn, B. 1977: Ultrastructure of the Cyst of *Giardia lamblia*. American Journal of Tropical Medicine and Hygiene **26**, 23-30.

Shinriki, N., Ishizaki, K., Yoshizaki, T., Miura, K. and Ueda, T. 1988: Mechanism of Inactivation of Tobacco Mosaic-Virus with Ozone. Water Research **22**, 933-938.

Siddiqui, M., Amy, G., Ozekin, K. and Westerhoff, P. 1998: Modeling Dissolved Ozone and Bromate Ion Formation in Ozone Contactors. Water Air and Soil Pollution **108**, 1-32.

Sikkema, J., de Bont, J. A. and Poolman, B. 1995: Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews **59**, 201-222.

Singer, P. C. 1993: Formation and Characterization of Disinfection By- products. Paper pres. Safety of water disinfection: balancing chemical and microbial risks, Washington.

Skidal'skaia, A. M. 1969: New data on the mechanism of disinfection of drinking water with chlorine and gamma irradiation. Gigiena i Sanitariia **34**, 11-17.

Snoeyink, V. L. and Jenkins, D. 1980: Water Chemistry. John Wiley and Sons, Inc., New York.

Sobsey, M. D. 1989: Inactivation of Health-Related Microorganisms in Water by Disinfection Processes. Water Science and Technology **21**, 179-195.

Soltys, B. J., Falah, M. and Gupta, R. S. 1996: Identification Of Endoplasmic Reticulum In the Primitive Eukaryote *Giardia lamblia* Using Cryoelectron Microscopy and Antibody to Bip. Journal Of Cell Science **7**, 1909-1917.

Soltys, B. J. and Gupta, R. S. 1994: Immunoelectron microscopy of *Giardia lamblia* cytoskeleton using antibody to acetylated alpha-tubulin. Journal of Eukaryotic Microbiology **41**, 625-632.

Sproul, O. J., Pfister, R. M. and Kim, C. K. 1982: The Mechanism of Ozone Inactivation of Water Borne Viruses. Water Science and Technology **14**, 303-314.

Staehelin, J. and Hoigné, J. 1982: Decomposition of Ozone in Water: Rate of Initiation by Hydroxide Ions and Hydrogen Peroxide. Environmental Science and Technology **16**, 676-681.

Staehelin, J. and Hoigné, J. 1985: Decomposition of Ozone in Water in the Presence of Organic Solutes Acting as Promoters and Inhibitors of Radical Chain Reactions. Environmental Science and Technology **19**, 1206-1213.

Sullivan, D. E. and Roth, J. A. 1980: Kinetics of Ozone Self-Decomposition in Aqueous Solution. In: Water-1979, Vol. 76 (Bennett, G. F. ed). American Institute of Chemical Engineers, New York, NY. pp. 142-149.

Sundermann, C. A., Lindsay, D. S. and Blagburn, B. L. 1987: Evaluation of Disinfectants for Ability to Kill Avian *Cryptosporidium* Oocysts. Companion Animal Practice **2**, 36-39.

Swanson, E. S., Cunningham, W. P. and Holman, R. T. 1977: Ultrastructure of Glandular Trichomes on Ovary of "*Cypripedium calceolus* (Orchidaceae). Plant Physiology **59**, 20-20.

Sykes, G. 1965: Disinfection and sterilization. E. and E.N. Spon., Ltd, London.

Taghi-Kilani, R., Gyürék, L. L., Millard, P. J., Finch, G. R. and Belosevic, M. 1996: Nucleic Acid Stains As Indicators Of *Giardia muris* Viability Following Cyst Inactivation. International Journal For Parasitology. **26**, 637-646.

Taghi-Kilani, R. and Sekla, L. 1987: Purification of *Cryptosporidium* oocysts and sporozoites by cesium chloride and percoll gradients. The American Journal of Tropical Medicine and Hygiene **36**, 505-508.

Takamoto, Y., Maeba, H. and Kamimura, M. 1992: Changes in Survival Rate Enzyme-Activities and in *Escherichia coli* with Ozone. Applied Microbiology and Biotechnology **37**, 393-395.

Tenno, K. M., Fujioka, R. S. and Loh, P. C. 1979: The mechanism of poliovirus inactivation by hypochlorous acid. In: Proceedings of the third conference on water chlorination: environmental impact and health effects. (Jolley, R., Brungs, W. A. and Cumming, R. B. eds). Ann Arbor Science Publishers, Inc., Chelsea, MI. pp. 665-675.

Tomiyasu, H., Fukutomi, H. and Gordon, G. 1985: Kinetics and Mechanism of Ozone Decomposition in Basic Aqueous Solution. Inorganic Chemistry **24**, 2962-2966.

U.S. Environmental Protection Agency. 1998: National Primary Drinking Water Regulations: Disinfectants and Disinfection Byproducts; Final Rule. Federal Register **63**, 69389-69476.

Van Cruchten, S. and Van den Broeck, W. 2002: Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anatomia Histologia Embryologia-Journal of Veterinary Medicine Series C **31**, 214-223.

Venkobachar, C., Iyengar, L. and Prabhakara Rao, V. S. 1975: Mechanism of Disinfection. Water Research **9**, 119-124.

Venkobachar, C. 1977: Mechanism of Disinfection: Effect of Chlorine on Cell Membrane Functions. Water Research **11**, 727-729.

Wallis, P. M., van Roodselaar, A., Neuwirth, M., Roach, P. D., Buchanan-Mappin, J. M. and Mack, H. 1990: Inactivation of *Giardia* Cysts in a Pilot Plant Using Chlorine Dioxide and Ozone. In: Proceedings of the American Water Works Association Water Quality Technology Conference, November 12-16,1989, Philadelphia, PA. American Water Works Association, Denver, CO. pp. 695-708.

Ward, H. D., Kane, A. V., Ortegabarria, E., Keusch, G. T. and Pereira, M. E. A. 1990: Identification of Developmentally Regulated *Giardia lamblia* Cyst Antigens Using Gcsa-1, a Cyst-Specific Monoclonal-Antibody. Molecular Microbiology **4**, 2095-2102.

Ward, W., Alvarado, L., Rawlings, N. D., Engel, J. C., Franklin, C. and McKerrow, J. H. 1997: A primitive enzyme for a primitive cell: The protease required for excystation of *Giardia*. Cell **89**, 437-444.

Watson, H. E. 1908: A Note on the Variation of the Rate of Disinfection with Change in the Concentration of the Disinfectant. Journal of Hygiene (Cambridge) **8**, 536-592.

White, G. C. 1992: Handbook of Chlorination and Alternative Disinfectants. Van Nostrand Reinhold Co., New York, NY.

Whiteside, C. and Hassan, H. M. 1988: Role of oxyradicals in the inactivation of catalase by ozone. Free Radical Biology and Medicine **5**, 305-312.

Wickramanayake, G. B. 1984: Kinetics and Mechanism of Ozone Inactivation of Protozoan Cysts. PhD dissertation, Ohio State University.

Wickramanayake, G. B., Rubin, A. J. and Sproul, O. J. 1984a: Inactivation of *Giardia lamblia* Cysts with Ozone. Applied and Environmental Microbiology **48**, 671-672.

Wickramanayake, G. B., Rubin, A. J. and Sproul, O. J. 1984b: Inactivation of *Naegleria* and *Giardia* Cysts in Water by Ozonation. Journal of the Water Pollution Control Federation **56**, 983-988.

Wickramanayake, G. B., Rubin, A. J. and Sproul, O. J. 1985: Effects of Ozone and Storage Temperature on *Giardia* Cysts. Journal of the American Water Works Association **77**, 74-77.

Wiesehahn, G. P., Jarroll, E. L., Lindmark, D. G., Meyer, E. A. and Hallick, L. M. 1984: *Giardia lamblia* - Autoradiographic Analysis of Nuclear Replication. Experimental Parasitology **58**, 94-100.

Williams, D. E., Swango, L. J,, Wilt, G. R. and Worley, S. D. 1991: Effect of Organic N-halamines on Selected Membrane Functions in Intact *Staphylococcus aureus* Cells. Applied and Environmental Microbiology **57**, 1121-1127.

Williams, N. D. and Russell, A. D. 1991: The Effects of Some Halogen-containing Compounds on *Bacillus subtilis* Endospores. Journal of Applied Bacteriology **70**, 427-436.

Wolfe, M. S. 1990: Clinical Symptoms and Diagnosis by Traditional Methods. In: Giardiasis, Vol. 3 (Meyer, E. A. ed). Elsevier, Amsterdam. pp. 176-185.

Wyllie, A. H. 1974: Death in normal and neoplastic cells. Journal of Clinical Pathology - supplement (Royal College of Pathologists) **7**, 35-42.

Wyllie, A. H. 1994: Death from inside out: an overview. Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences **345**, 237-241.

Yoshizaki, T., Miura, K. and Ueda, T. 1988: Mechanism of inactivation of tobacco mosaic virus with ozone. Water Resources **22**, 933-938.

Young, D. C. and Sharp, D. G. 1985: Virion Conformational Forms and the Complex Inactivation Kinetics of Echovirus by Chlorine in Water. Applied and Environmental Microbiology **49**, 359-364.

Zaske, S. K., Dockins, W. S. and McFeters, G. A. 1980: Cell-Envelope Damage in *Escherichia coli* Caused by Short-Term Stress in Water. Applied and Environmental Microbiology **40**, 386-390.

APPENDIX A
CONCENTRATION AND CONTACT TIME PROFILE FOR SINGLE CHEMICAL EXPERIMENTAL TRIALS

Table A-1. Concentration and contact time profile for ozone trials

Trial	Date	Temp. °C	pH	Reactor Vol mL	Average Stock Conc mg/L	Stock Vol added mL	Applied Dose mg/L	Initial Residual mg/L	Final Residual mg/L	Average Residual mg/L	Contact Time min	Average CT mg/L*min	Observed kill Log-units
1*	26-Nov-96	25	6	200	21.28	1.00	0.1	0.1	0.1	0.1	0.5	0.05	0.30
2*	26-Nov-96	25	6	200	20.76	5.00	0.5	0.5	0.4	0.5	5	2.3	>2
3*	1-Dec-96	25	6	200	21.23	1.00	0.1	0.1	0.1	0.1	0.5	0.05	0.42
4h*	1-Dec-96	25	6	200	21.98	4.60	0.5	0.5	0.4	0.5	2	0.9	1.19
5*	16-Jan-97	25	6	200	22.45	4.50	0.5	0.5	0.5	0.5	2	0.9	1.39
6*	16-Jan-97	25	6	200	22.08	4.60	0.5	0.5	0.4	0.4	5	2.2	1.92
1	19-Aug-02	5	6	100	38.20	0.51	0.2	0.2	0.2	0.2	1	0.2	0.41
2	21-Aug-02	5	6	150	42.27	0.70	0.2	0.2	0.2	0.2	1	0.2	0.28
3	1-Sep-02	5	6	100	38.08	1.30	0.5	0.5	0.4	0.5	5	2.3	>2
4	7-Sep-02	5	6	100	41.37	1.19	0.5	0.5	0.4	0.5	5	2.3	1.20
5	9-Sep-02	5	6	100	42.17	0.47	0.2	0.2	0.2	0.2	1	0.2	0.37
6	14-Sep-02	5	6	100	33.45	0.57	0.2	0.2	0.2	0.2	1	0.2	0.32
7	14-Sep-02	5	6	100	37.17	0.51	0.2	0.2	0.2	0.2	1	0.2	0.39
8	15-Sep-02	5	6	100	29.10	1.69	0.5	0.5	0.4	0.4	5	2.2	1.51
9	15-Sep-02	5	6	100	24.02	2.00	0.5	0.5	0.4	0.5	5	2.3	1.30

Table A-1. Continued

Trial	Control				Test				Observed kill Log-units
	Green	Red	Total	% Viable	Green	Red	Total	%Viable	
1*	498	130	628	0.79	139	209	348	0.40	0.3
2*	498	130	628	0.79	0	269	269	0.00	>2
3*	365	95	460	0.79	102	237	339	0.30	0.4
4h*	365	95	460	0.79	20	372	392	0.05	1.2
5*	345	78	423	0.82	15	438	453	0.03	1.4
6*	345	78	423	0.82	2	203	205	0.01	1.9
1	324	40	364	0.89	203	387	590	0.34	0.4
2	257	78	335	0.77	141	208	349	0.40	0.3
3	300	81	381	0.79	0	205	205	0.00	>2
4	357	101	458	0.78	11	211	222	0.05	1.2
5	330	77	407	0.81	122	232	354	0.34	0.4
6	347	92	439	0.79	132	217	349	0.38	0.3
7	347	92	439	0.79	132	278	410	0.32	0.4
8	356	96	452	0.79	3	121	124	0.02	1.5
9	356	96	452	0.79	9	220	229	0.04	1.3

Table A-2. Concentration and contact time profile for free chlorine trials

Trial	Date	Temp. °C	pH	Reactor Vol mL	Average Stock Conc mg/L	Stock Vol added mL	Applied Dose mg/L	Initial Residual mg/L	Final Residual mg/L	Average Residual mg/L	Contact Time min	Average CT mg/L*min	Observed kill Log-units
7*	11-Feb-97	25	6	200	143.25	2.83	2.00	2.0	1.6	1.8	30	54	0.40
8*	11-Feb-97	25	6	200	143.25	2.83	2.00	1.9	1.7	1.8	100	180	0.88
9*	11-Feb-97	25	6	200	143.25	7.20	4.98	5.0	4.8	4.9	120	586	1.63
10*	27-Feb-97	25	6	200	143.25	2.83	2.00	2.1	1.9	2.0	30	60	0.48
11*	27-Feb-97	25	6	200	143.25	2.83	2.00	2.0	1.9	1.9	100	194	1.21
12*	27-Feb-97	25	6	200	143.25	7.20	4.98	4.9	4.5	4.7	120	560	1.70
1	19-Aug-02	5	6	50	30.35	3.29	1.87	1.9	1.7	1.8	90	160	0.16
2	21-Aug-02	5	6	100	73.69	6.79	4.69	4.7	4.5	4.6	30	138	0.16
2	21-Aug-02	5	6	100	73.69	6.79	4.69	4.8	4.2	4.5	120	542	0.60
3	1-Sep-02	5	6	100	64.31	7.57	4.53	5.0	4.4	4.7	120	559	0.70
4	7-Sep-02	5	6	100	230.96	0.87	1.99	2.0	1.8	1.9	30	58	0.25
5	9-Sep-02	5	6	100	205.05	0.98	1.99	2.0	1.9	1.9	30	58	0.19
6	14-Sep-02	5	6	100	205.05	0.98	1.99	1.9	1.9	1.9	30	57	0.21
7	14-Sep-02	5	6	100	205.05	2.44	4.88	4.8	4.5	4.6	166	768	0.85
8	15-Sep-02	5	6	100	205.05	0.98	1.99	2.0	1.9	1.9	30	58	0.19
9	15-Sep-02	5	6	100	205.05	2.44	4.88	4.9	4.3	4.6	120	549	0.81

Table A-2. Continued

Trial	Control				Test				Observed kill
	Green	Red	Total	% Viable	Green	Red	Total	%Viable	Log-units
7*	301	99	400	0.75	109	253	362	0.30	0.4
8*	301	99	400	0.75	36	323	359	0.10	0.9
9*	301	99	400	0.75	3	167	170	0.02	1.6
10*	363	74	437	0.83	98	258	356	0.28	0.5
11*	363	74	437	0.83	12	224	236	0.05	1.2
12*	363	74	437	0.83	4	238	242	0.02	1.7
1	324	40	364	0.89	265	165	430	0.62	0.2
2	257	78	335	0.77	255	222	477	0.53	0.2
2	257	78	335	0.77	50	209	259	0.19	0.6
3	300	81	381	0.79	39	209	248	0.16	0.7
4	357	101	458	0.78	118	149	267	0.44	0.2
5	330	77	407	0.81	183	168	351	0.52	0.2
6	347	92	439	0.79	153	158	311	0.49	0.2
7	347	92	439	0.79	31	245	276	0.11	0.8
8	356	96	452	0.79	59	56	115	0.51	0.2
9	356	96	452	0.79	34	243	277	0.12	0.8

APPENDIX B

CONCENTRATION AND CONTACT TIME PROFILE FOR MULTIPLE CHEMICAL EXPERIMENTAL TRIALS

Table B-1. Concentration and contact time profile for multiple chemical trials

Experimental Design	Trial	Date	Temp. °C	pH	1st Disinfectant									
					Type	Reactor Vol mL	Average Stock Conc mg/L	Stock Vol added mL	Applied Dose mg/L	Initial Residual mg/L	Final Residual mg/L	Average Residual mg/L	Contact Time min	Average CT mg/L*min
low ozone, low chlorine	1	19-Aug-02	5	6	Ozone	100	38.20	0.51	0.2	0.2	0.2	0.2	1	0.2
low ozone, low chlorine	2a1	21-Aug-02	5	6	Ozone	150	42.27	0.70	0.2	0.2	0.2	0.2	1	0.2
low chlorine, low ozone	2b1	21-Aug-02	5	6	Chlorine	100	73.69	6.79	4.7	4.8	4.4	4.6	30	138
low ozone, moderate chlorine	2a2	21-Aug-02	5	6	Ozone	150	42.27	0.70	0.2	0.2	0.2	0.2	1	0.2
moderate chlorine, low ozone	2b2	21-Aug-02	5	6	Chlorine	100	73.69	6.79	4.7	4.6	4.2	4.4	120	530
moderate ozone, moderate chlorine	3a	1-Sep-02	5	6	Ozone	100	38.08	1.30	0.5	0.5	0.4	0.5	5	2.3
moderate chlorine, moderate ozone	3b	1-Sep-02	5	6	Chlorine	100	64.31	7.57	4.5	4.5	4.3	4.4	120	528
moderate ozone, low chlorine	4a	7-Sep-02	5	6	Ozone	100	41.37	1.19	0.5	0.5	0.4	0.5	5	2.3
low chlorine, moderate ozone	4b	7-Sep-02	5	6	Chlorine	100	230.96	0.87	2.0	2.0	1.8	1.9	30	58
low ozone, low chlorine	5a	9-Sep-02	5	6	Ozone	100	42.17	0.47	0.2	0.2	0.2	0.2	1	0.2
low chlorine, low ozone	5b	9-Sep-02	5	6	Chlorine	100	205.05	0.98	2.0	2.0	1.7	1.9	30	56
low ozone, low chlorine	6a	14-Sep-02	5	6	Ozone	100	33.45	0.57	0.2	0.2	0.2	0.2	1	0.2
low chlorine, low ozone	6b	14-Sep-02	5	6	Chlorine	100	205.05	0.98	2.0	1.9	1.9	1.9	30	57
low ozone, moderate chlorine	7a	14-Sep-02	5	6	Ozone	100	37.17	0.51	0.2	0.2	0.2	0.2	1	0.2
moderate chlorine, low ozone	7b	14-Sep-02	5	6	Chlorine	100	205.05	2.44	4.9	4.8	4.6	4.7	166	776
moderate ozone, low chlorine	8a	15-Sep-02	5	6	Ozone	100	29.10	1.69	0.5	0.5	0.4	0.4	5	2.2
low chlorine, moderate ozone	8b	15-Sep-02	5	6	Chlorine	100	205.05	0.98	2.0	2.0	1.9	1.9	30	58
moderate ozone, moderate chlorine	9a	15-Sep-02	5	6	Ozone	100	24.02	2.00	0.5	0.5	0.4	0.5	5	2.3
moderate chlorine, moderate ozone	9b	15-Sep-02	5	6	Chlorine	100	205.05	2.44	4.9	4.8	4.5	4.7	120	558

Table B-1. Continued

2nd Disinfectant										Sequential Treatment									1st Disinfectant Alone	2nd Disinfectant Alone	Additive kill	Synergy
										Control				Test								
Type	Reactor Vol	Average Stock Conc	Stock Vol added	Applied Dose	Initial Residual	Final Residual	Average Residual	Contact Time	Average CT	Green	Red	Total	% Viable	Green	Red	Total	% Viable	Observed kill	Observed kill	Observed kill		
	mL	mg/L	mL	mg/L	mg/L	mg/L	mg/L	min	mg/L*min									Log-units	Log-units	Log-units	Log-units	Log-units
Chlorine	50	30.35	3.29	1.9	1.9	1.7	1.8	90	160	324	40	364	0.89	74	325	399	0.19	0.7	0.4	0.2	0.6	0.1
Chlorine	100	73.69	6.79	4.7	4.8	4.4	4.6	30	138	257	78	335	0.77	73	238	311	0.23	0.5	0.3	0.2	0.4	0.1
Ozone	150	42.27	0.70	0.2	0.2	0.2	0.2	1	0.2	257	78	335	0.77	25	379	404	0.06	1.1	0.2	0.3	0.4	0.7
Chlorine	100	73.69	6.79	4.7	4.6	4.2	4.4	120	530	257	78	335	0.77	9	245	254	0.04	1.3	0.3	0.6	0.9	0.5
Ozone	150	42.27	0.70	0.2	0.2	0.2	0.2	1	0.2	257	78	335	0.77	2	232	234	0.01	2.0	0.6	0.3	0.9	1.1
Chlorine	55	64.31	4.20	4.6	4.6	4.5	4.6	120	546	300	81	381	0.79	0	266	266	0.00	> 2	>2	0.7	> 2.7	?
Ozone	100	38.08	1.30	0.5	0.5	0.4	0.5	5	2.3	300	81	381	0.79	0	205	205	0.00	>2	0.7	>2	> 2.7	?
Chlorine	55	230.96	0.48	2.0	2.0	1.8	1.9	30	57	357	101	458	0.78	0	220	220	0.00	> 2	1.2	0.2	1.4	> 0.6
Ozone	100	41.37	1.19	0.5	0.5	0.4	0.5	5	2.3	357	101	458	0.78	0	211	211	0.00	> 2	0.2	1.2	1.4	> 0.6
Chlorine	55	205.05	0.54	2.0	2.0	1.7	1.9	30	56	330	77	407	0.81	95	384	479	0.20	0.6	0.4	0.2	0.6	0.0
Ozone	100	42.17	0.47	0.2	0.2	0.2	0.2	1	0.2	330	77	407	0.81	n/a	n/a	n/a	n/a	n/a	0.2	0.4	0.6	n/a
Chlorine	80	205.05	0.78	2.0	1.9	1.6	1.8	30	53	347	92	439	0.79	47	176	223	0.21	0.6	0.3	0.2	0.5	0.0
Ozone	70	31.35	0.42	0.2	0.2	0.2	0.2	1	0.2	347	92	439	0.79	30	255	285	0.11	0.9	0.2	0.3	0.5	0.3
Chlorine	80	205.05	1.95	4.9	4.8	4.7	4.7	166	784	347	92	439	0.79	3	150	153	0.02	1.6	0.4	0.8	1.2	0.4
Ozone	70	35.48	0.38	0.2	0.2	0.2	0.2	1	0.2	347	92	439	0.79	0	257	257	0.00	> 2	0.8	0.4	1.2	> 0.8
Chlorine	80	205.05	0.78	2.0	1.9	1.9	1.9	30	57	356	96	452	0.79	0	289	289	0.00	> 2	1.5	0.2	1.7	> 0.3
Ozone	70	30.72	1.10	0.5	0.5	0.4	0.4	5	2.2	356	96	452	0.79	2	232	234	0.01	2.0	0.2	1.5	1.7	0.3
Chlorine	80	205.05	1.95	4.9	4.9	4.6	4.7	120	567	356	96	452	0.79	0	278	278	0.00	> 2	1.3	0.8	2.1	?
Ozone	70	33.53	1.03	0.5	0.5	0.4	0.5	5	2.3	356	96	452	0.79	0	221	221	0.00	> 2	0.8	1.3	2.1	?

APPENDIX C

SUMMARY OF DOSE RESPONSE DATA FOR SINGLE CHEMICAL EXPERIMENTAL TRIALS

Table C-1. Summary of dose response data for single chemical experiments

Type of Disinfectant	Experimental Design	Trial	Temp. °C	pH	Applied Dose mg/L	Average Residual mg/L	Contact Time min	Average CT mg/L*min	Observed kill Log-units	Observed kill Log-units
Ozone	Low	1*	25	6	0.1	0.1	0.5	0.05	0.30	0.3
		3*	25	6	0.1	0.1	0.5	0.05	0.42	0.4
	Moderate	4h*	25	6	0.5	0.5	2	0.9	1.19	1.2
		5*	25	6	0.5	0.5	2	0.9	1.39	1.4
	High	6*	25	6	0.5	0.4	5	2.2	1.92	1.9
		2*	25	6	0.5	0.5	5	2.3	>2	>2
	Low	5	5	6	0.2	0.2	1	0.2	0.37	0.4
		7	5	6	0.2	0.2	1	0.2	0.39	0.4
		2	5	6	0.2	0.2	1	0.2	0.28	0.3
		6	5	6	0.2	0.2	1	0.2	0.32	0.3
		1	5	6	0.2	0.2	1	0.2	0.41	0.4
	Moderate	8	5	6	0.5	0.4	5	2.2	1.51	1.5
		9	5	6	0.5	0.5	5	2.3	1.30	1.3
		3	5	6	0.5	0.5	5	2.3	>2	>2
		4	5	6	0.5	0.5	5	2.3	1.20	1.2
Free Chlorine	Low	7*	25	6	2.00	1.8	30	54	0.40	0.4
		10*	25	6	2.00	2.0	30	60	0.48	0.5
	Moderate	8*	25	6	2.00	1.8	100	180	0.88	0.9
		11*	25	6	2.00	1.9	100	194	1.21	1.2
	High	9*	25	6	4.98	4.9	120	586	1.63	1.6
		12*	25	6	4.98	4.7	120	560	1.70	1.7
	Low	2	5	6	4.69	4.6	30	138	0.16	0.2
		1	5	6	1.87	1.8	90	160	0.16	0.2
		8	5	6	1.99	1.9	30	58	0.19	0.2
		5	5	6	1.99	1.9	30	58	0.19	0.2
		6	5	6	1.99	1.9	30	57	0.21	0.2
		4	5	6	1.99	1.9	30	58	0.25	0.2
	Moderate	2	5	6	4.69	4.5	120	542	0.60	0.6
		3	5	6	4.53	4.7	120	559	0.70	0.7
		9	5	6	4.88	4.6	120	549	0.81	0.8
		7	5	6	4.88	4.6	166	768	0.85	0.8

APPENDIX D

SUMMARY OF DOSE RESPONSE DATA FOR SINGLE CHEMICAL EXPERIMENTAL TRIALS

Table D-1. Summary of dose response data for multiple chemical experiments

Experimental Design	Trial	Temp. °C	pH	1st Disinfectant Type	1st Disinfectant Applied Dose mg/L	1st Disinfectant Average Residual mg/L	1st Disinfectant Contact Time min	1st Disinfectant Average CT mg/L*min	2nd Disinfectant Type	2nd Disinfectant Applied Dose mg/L	2nd Disinfectant Average Residual mg/L	2nd Disinfectant Contact Time min	2nd Disinfectant Average CT mg/L*min	Sequential Treatment Observed kill Log-units	1st Disinfectant Alone Observed kill Log-units	2nd Disinfectant Alone Observed kill Log-units	Additive kill Log-units	Synergy Log-units
low ozone, low chlorine	6a	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	2.0	1.8	30	53	0.6	0.3	0.2	0.5	0.0
	5a	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	2.0	1.9	30	56	0.6	0.4	0.2	0.6	0.0
	2a1	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	4.7	4.6	30	138	0.5	0.3	0.2	0.4	0.1
	1	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	1.9	1.8	90	160	0.7	0.4	0.2	0.6	0.1
low ozone, moderate chlorine	2a2	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	4.7	4.4	120	530	1.3	0.3	0.6	0.9	0.5
	7a	5	6	Ozone	0.2	0.2	1	0.2	Chlorine	4.9	4.7	166	784	1.6	0.4	0.8	1.2	0.4
moderate ozone, low chlorine	8a	5	6	Ozone	0.5	0.4	5	2.2	Chlorine	2.0	1.9	30	57	> 2	1.5	0.2	1.7	> 0.3
	4a	5	6	Ozone	0.5	0.5	5	2.3	Chlorine	2.0	1.9	30	57	> 2	1.2	0.2	1.4	> 0.6
moderate ozone, moderate chlorine	3a	5	6	Ozone	0.5	0.5	5	2.3	Chlorine	4.6	4.6	120	546	> 2	>2	0.7	> 2.7	?
	9a	5	6	Ozone	0.5	0.5	5	2.3	Chlorine	4.9	4.7	120	567	> 2	1.3	0.8	2.1	?
low chlorine, low ozone	5b	5	6	Chlorine	2.0	1.9	30	56	Ozone	0.2	0.2	1	0.2	n/a	0.2	0.4	0.6	n/a
	6b	5	6	Chlorine	2.0	1.9	30	57	Ozone	0.2	0.2	1	0.2	0.9	0.2	0.3	0.5	0.4
	2b1	5	6	Chlorine	4.7	4.6	30	138	Ozone	0.2	0.2	1	0.2	1.1	0.2	0.3	0.5	0.6
low chlorine, moderate ozone	8b	5	6	Chlorine	2.0	1.9	30	58	Ozone	0.5	0.4	5	2.2	2.0	0.2	1.5	1.7	0.3
	4b	5	6	Chlorine	2.0	1.9	30	58	Ozone	0.5	0.5	5	2.3	> 2	0.2	1.2	1.4	> 0.6
moderate chlorine, low ozone	7b	5	6	Chlorine	4.9	4.7	166	776	Ozone	0.2	0.2	1	0.2	> 2	0.8	0.4	1.2	> 0.8
	2b2	5	6	Chlorine	4.7	4.4	120	530	Ozone	0.2	0.2	1	0.2	2.0	0.6	0.3	0.9	1.1
moderate chlorine, moderate ozone	9b	5	6	Chlorine	4.9	4.7	120	558	Ozone	0.5	0.5	5	2.3	> 2	0.8	1.3	2.1	?
	3b	5	6	Chlorine	4.5	4.4	120	528	Ozone	0.5	0.5	5	2.3	>2	0.7	> 2	> 2.7	?